FABRICATION AND IN-VITRO CHARACTERIZATION OF ELECTROSPUN POLYCAPROLACTONE MATS CONTAINING BIOACTIVE CALCIUM SULFATE FOR PERIODONTAL TISSUE REGENERATION

DISSERTATION

Submitted to The Tamil Nadu Dr. M.G.R Medical University
in partial fulfillment of the requirement for the degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

2015 - 2018

CERTIFICATE

This is to certify that the dissertation titled entitled **“Fabrication and in-vitro characterization of electrospun polycaprolactone mats containing bioactive calcium sulfate for periodontal tissue regeneration"** is a bonafide record of the work done by **Dr. Anju Unnikrishnan,** under our guidance during his postgraduate study period of 2015-2018. The dissertation is submitted to **THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY, CHENNAI,** in partial fulfillment of the requirement for the Degree of **MASTER OF DENTAL SURGERY IN PERIODONTOLOGY, BRANCH II.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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I hereby declare that this dissertation, entitled "**Fabrication and in-vitro characterization of electrospun polycaprolactone mats containing bioactive calcium sulfate for periodontal tissue regeneration**", is a bonafide record of work undertaken by me and that this thesis or a part of it has not been presented earlier for the award of degree, diploma, fellowship, or similar title of recognition.

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‘‘There is no substitute for hardwork’’ - Thomas A Edison

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CONTENTS

LIST OF ABBREVIATIONS

PCL	-	Polycaprolactone
CaS	-	Calcium Sulfate
BioCaS	-	Bioactive calcium sulfate
DMSO	-	Dimethylsulfoxide
THF	-	Tetrahydrofuran
E-spinning	-	Electrospinning
ECM	-	Extracellular Matrix
CP	-	Calcium Phosphate
TiO_2	-	Titanium Dioxide
PGA	-	Polyglycolic acid
BGM	-	Bio glass microspheres
rMSCs	-	Rat mesenchymal stem cells
FTIR	-	Fourier Transformation Infrared Spectroscopy
GTR	-	Guided Tissue Regeneration
Kv	-	Kilovolt
µg	-	Microgram
mg	-	Milligram
mm	-	Millimetre
Mwt	-	Molecular weight
OD	-	Optical Density
PDL	-	Periodontal Ligament
PBS	-	Phosphate Buffered saline

PCL+ CaS 5%	-	Polycaprolactone blended with 5% of Bioactive Calcium sulfate
PCL+ CaS 10%	-	Polycaprolactone blended with 10% of Bioactive Calcium sulfate
PGA	-	Polyglycolic acid
SEM	-	Scanning Electron Microscope
SPSS	-	Statistical package for social sciences
3D	-	Three Dimensional
TE	-	Tissue Engineering

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CP No	Title
CP 1	Polycaprolactone (PCL) pellets with M_{WT} 80,000 (sigma Aldrich Pt Ltd, USA
CP 2	Bioactive Calcium sulfate
CP 3	Dimethylsulfoxide DMSO (C_2H_6OS),minimum 99% were purchased from spectrochem PVT.LTD, Mumbai,India
CP 4	Tetrahydrofuran Tetrahydrofuran (THF – packed under nitrogen C_4H_8O, minimum 99.9%) from spectrochem PVT.LTD, Mumbai,India
CP 5	PCL incorporated with bioactive CaS
CP 6	Electrospinning unit (Holmarc-USA) -Division of Tissue Culture, BMT wing, Sree Chitra Tirunal institute of medical sciences and technology
CP 7	Syringe Pump
CP 8	Electronic weighing machine
CP 9	Thickness gauge
CP 10	Automatic vernier caliper
CP 11	FTIR Spectrometer
CP 12	Specimen prepared for SEM analysis
CP 13	Data physics OCA 15Plus for suface wettability

CP 14	Homogenizer (Polytron –PT 1600E)
CP 15	Universal testing machine (Instron 3345,single column, UK)
CP 16	Specimen prepared for direct contact assay
CP 17	Specimen prepared for cell adhesion
CP 18	Phase contrast microscope

LIST OF ANNEXURES

ABSTRACT

Background

Periodontitis is an inflammatory disease of supporting tissues of teeth caused by specific microorganisms resulting in progressive destruction of periodontal ligament and alveolar bone with pocket formation, recession or both. The main consequences of periodontal disease are periodontal destruction, alveolar bone loss leading to loosening or loss of teeth. It is caused by microbial plaque deposition and host microbial interaction. The main treatment approaches are non-surgical and surgical debridement. Surgical periodontal therapy includes Resective and Regenerative procedures. To date, flap debridement or flap curettage and periodontal regenerative therapy with membranes and bone substitute materials have been employed with distinct levels of clinical success.

The developing field of tissue engineering aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue. Guided Tissue Regeneration is the method for the prevention of epithelial migration along the cemental wall of the pocket and maintaining space for clot stabilization. Synthetic polymers have excellent design flexibility because their composition and structure can be tailored to the specific applications. Tissue engineering has emerged as a promising solution in treating extensive loss/damage of skin caused by burns, trauma and diseases.

Objective

i. To fabricate third generation GTR membrane containing synthetic polymer Polycaprolactonewith varying concentration of calcium sulphate.

ii. In-vitro characterization of the material- To study the morphological, mechanical, chemical properties and biocompatibility testing.

Materials and Methods

GTR membranes made of polycaprolactone with a molecular weight of 80,000 reinforced with varying concentration of bioactive calcium sulphate (5% and 10%) is fabricated by the method of Electrospinning. After fabrication, their invitro properties are evaluated. Morphology of the membranes such as fibre diameter and fibre density was evaluated by SEM(Scanning electrone microscope), mechanical properties such as tensile strength, suture pull out using Instron 3345 universal testing machine and water contact angle using Data physics OCA 15plus by sessile drop method .For chemical properties FTIR analysis was performed. And finally cytocompatibility analysis were evaluated using L-929 mouse fibroblast by Direct contact assay and Cell adhesion study.

Results

All the electrospun nanofibrous membranes possessed appropriate mechanical as well as chemical properties. Moreover, none of the membranes found to be cytotoxic. On comparing the overall properties, PCL + 10% CaS exhibited superior cell adhesion and mechanical as well as chemical properties which satisfies the ideal properties needed for GTR membranes.

Conclusion

PCL-CaS blend was prepared in a solvent of THF and DMSO and fabricated by employing electrospinning technique.In the present study the results showed that composite electrospun scaffolds (PCL-CaS) were superior to PCL scaffolds alone. Based on the above results, we conclude that the electrospun mat fabricated with PCL incorporated with medical grade Calcium sulfate employed by electrospinning technique is suitable for periodontal tissue engineering. And further animal experiments followed by human clinical trials need to be evaluated for future applications.

Keywords:

Periodontal tissue regeneration, Polycarpolactone, Bioactive calcium sulphate, Electrospinning, Electrospunscaffold.

INTRODUCTION

Periodontitis is a multifactorial disease of supporting tissues of teeth caused by specific microorganisms resulting in progressive destruction of periodontal ligament and alveolar bone with pocket formation, recession or both. The main consequences of periodontal disease are periodontal destruction, alveolar bone loss leading to loosening or loss of teeth. It is caused by microbial plaque deposition and host microbial interaction. The main treatment approaches are non-surgical and surgical debridement. Surgical periodontal therapy includes resective and regenerative procedures.

Healing is a cell response to injury in an attempt to restore the normal structure and function. It involves two distinct processes Regeneration and Repair which occurs simultaneously. Bone possesses the intrinsic capacity for regeneration as part of the repair process in response to injury, as well as during skeletal development or continuous remodelling throughout adult life.[1]Bone regeneration is comprised of a well-orchestrated series of biological events of bone induction and conduction, involving a number of cell types and intracellular and extracellular molecular signalling pathways. [1] The developing field of tissue engineering aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue. Guided Tissue Regeneration is the method for the prevention of epithelial migration along the cemental wall of the pocket and maintaining space for clot stabilization. They are either resorbable & non - resorbable. The main approaches of regeneration are growth factors, Tissue Engineering and Gene Therapy.

GTR membranes as physical barriers however provide no biological effects on differentiation and proliferation of mesenchyme and the periodontal ligament. According to Gottlow's classification; GTR membranes are classified to three generations Non-bioresorbable, Resorbable and Bioresorbable with Bioactive materials. Third generation membranes are used nowadays. Synthetic polymers have excellent design flexibility because their composition and structure can be tailored to the specific applications. Tissue engineering has emerged as a promising solution in treating extensive loss/damage of skin caused by burns, trauma and diseases. Critical determinants in favourable wound healing outcomes are largely based on the physicochemical nature of scaffolds, cell types and cell material interactions.[2]Biodegradable natural and synthetic polymers with favourable mechanical properties and degradation kinetics are extensively explored for therapeutic applications. From the list of synthetic bioresorbable polymers, polycaprolactone has drawn a great deal of attention in the past several years. This particular polymer has been successfully incorporated as an implantable biomaterial for medical applications including sutures and wound dressing. cardiovascular engineering, nerve regeneration, and bone tissue engineering.[3]

Polycaprolactone (PCL) is a semi-synthetic polymer, widely used for biomedical as well as dental applications due to its properties like low melting point, non-toxic nature, high tensile strength and biodegradability.[4] PCL has been investigated as potential matrix for skin tissue engineering. However use of PCL as such in tissue engineering is limited due to its poor bioregulatory activity, high hydrophobicity, lack of functional groups and neutral charge.[5] Various physico-chemical and post-processing surface modification techniques have been reported to

overcome these limitations.[6] Blending another polymer with desired characteristics will enable to bring down the disadvantages of PCL.[7]In this work we focused on another calcium salt, calcium sulfate, which is one of the synthetic materials with the longest clinical history of use in orthopaedics and dentistry as an implant for filling bone defects. It has been utilized in periodontal disease, endodontic lesions, alveolar bone loss and maxillary sinus augmentation.[8] It is well tolerated by the tissues, resorbable, acts as a space maintainer that prevents soft tissue from invading the defect until bone can grow in and proved very osteogenic in vivo.[9,10,11] Calcium sulfate is generally employed in the hemi-hydrate form ($CaSO_4$.½ H_2O or CHS) which is transformed, by reaction with water, in the hard di-hydrate one ($CaSO_4$ .2 H_2O or gypsum). Notwithstanding all these advantageous properties, calcium sulfate (gypsum) has also disadvantage which includes brittleness and fast resorption rate limit its applications in bone regeneration techniques. In order to overcome these drawbacks, we developed a novel calcium sulfate /polymer system permitting to produce, into the bone defect, gypsum encapsulated in a biodegradable polymeric network. This strategy should prolong structural integrity and slow-down resorption of calcium sulfate whilst maintaining its properties. The polymer used to realize the network was PCL, a biodegradable and non cytotoxic polyester.[12]

Electrospinning is a unique method for the fabrication of biomimetic scaffolds with micro to nano scale topography mocking natural extracellular matrix (ECM) for favourable cell attachment and proliferation. It is one of the common method to produce fibrous substrates from natural and synthetic polymers for tissue engineering applications. Ideally, a substrate should satirize both the form and functionality of the native extracellular matrix (ECM). It is known that morphology,

architecture, and surface properties have strong influence on cell growth, spreading, activity, and functionality. Hence, scaffolds formed by electrospinning are gaining increasing interest due to their favourable properties providing ideal microenvironments.

Electrospinning is a versatile processing method used to produce very fine fibers. The history of this processing method to produce polymer fibers marked since 1934, when patented for preparing artificial threads by formhals. The basic mechanism of this process involves an application of a high electric potential (10–30 kV) to a polymer melt or solution across a finite distance between a conductive needle and a grounded collector. Electrically charged polymer solution forms a conical shape, called a Taylor cone which is seen at the tip of the needle. The needle tip overcomes the force due to surface tension and viscosity when is is connected to an electric charge, a whipping polymer jet is spew out from the apex of the cone. Finally a randomly oriented non-woven fabric will be formed when the jet is directed towards the ground collector, during when the solvent evaporates and fibers get deposit on the collector. The charged polymer jet travels in a straight manner for a short distance before undergoing the distortion and lashing instability movements, which in turn contribute to the narrowing of fiber. Certain parameters such as high voltage, feed rate, etc and polymer solution properties like viscosity, conductivity, etc play a vital role in changing fiber sizes and characteristics.[13]

The aim of the study was to fabricate third generation GTR membrane containing synthetic polymer PCL with varying concentration of calcium sulfate and in-vitro characterization of the material includes the morphological, mechanical, chemical properties and biocompatibility testing as well.

AIMS & OBJECTIVES

i. To fabricate third generation GTR membrane containing synthetic polymer polycaprolactone with varying concentration of calcium sulfate.

ii. In-vitro characterization of the material

- To study the morphological, mechanical, chemical properties and biocompatibility testing.

REVIEW OF LITERATURE

Tissue-engineered scaffolds have played a decisive role in the repair and regeneration of a diverse range of tissues, including bone. These scaffolds not only provide a supporting matrix for cells especially in bone tissue engineering, but also provide essential environments for cells to spread, migrate, multiply, and conform to differentiation into specific lineage. For this, scaffolds should be tuned physico-chemically, to successfully repair and regenerate tissue. Unlike conventional scaffolds that temporarily fill defects and need secondary surgery for their replacement and/or removal, promisingly therapeutic scaffolds have utilized a variety of biological actions that favour and trigger cells, especially stem cells, to carry out relevant therapeutic roles. Bone repair or regeneration is a part of a complex dynamic event that involves many molecules and cells. After scaffold implantation, the therapeutic actions should thus be harmonized with the biological events and even facilitate a better healing process. Key events in the active healing process include mild inflammatory reactions with no tissue rejection, substantial angiogenesis to form blood vessels, recruitment of progenitor/stem cells, and driving these cells toward osteogenic lineage and finalizing matrix maturation.[1]

Within the class of synthetic biodegradable polymers, polycaprolactone (PCL) has drawn a great deal of attention in the past several years. This particular polymer has been successfully incorporated as an implantable biomaterial for medical applications, including sutures and wound dressing, cardiovascular tissue engineering, nerve regeneration, and bone tissue engineering. Its use as a vehicle for controlled delivery of therapeutic molecules has also been extensively explored. PCL is a linear aliphatic polyester. It is a hydrophobic, semicrystalline, biocompatible, and relatively slow degrading polymer, which has been widely used

in the biomedical field for the last few decades. It is a thermoplastic polymer with several desirable features, including good stability under ambient conditions, ease of processaibility (thermal and solution), and has already been approved for use in a few products by the U.S. Food and Drug Administration.[14]

Calvo et al in 1996[15] further concluded that the colloidal nature of PCL nanosphere and nanocapsule carriers was the main factor responsible for favourable corneal transport, and that cornea penetration was not increased by variation of the inner structure or composition of the carriers. This finding was also observed by Marchal-Heussler et al. in their use of PCL as a colloidal nanoparticle suspension containing cartelol, finding the inner oily core of the carrier provided better cartelol entrapment and a more pronounced effect on intraocular pressure compared with cartelol eye drops.

Gatta L.A et al in 2005[4] stated that novel poly(e-caprolactone)/calcium sulphate system was prepared and characterized in order to enhance calcium sulfate (gypsum) performance as bone graft substitute overcoming its brittleness and fast resorption rate. A poly(e-caprolactone) (PCL) photo-crosslinkable derivative (PCL_f) was synthesized by reaction of a low molecular weight PCL diol with methacryloyl chloride and confirmed by FT-IR and ^1H NMR analyses. An injectable and easy mouldable mixture of PCL_f and calcium sulfate hemi-hydrate (PCL_f/CHS) was obtained. Thermal analyses and solvent extraction proved the occurrence of PCL_fphoto-crosslinking, even in the presence of CHS, in a time suitable for clinical applications. Swelling studies demonstrated that the encapsulation of the inorganic filler increases network hydrophilicity making it more permeable to water. Scanning

electron microscopy, performed on crosslinkedPCL_f/CHS and on the same material after incubation in a PBS solution, showed the feasibility to obtain, in situ, gypsum entrapped into a degradable polymeric network. In vitro cytotoxicity tests, performed according to ISO 10993-5, proved that the developed system was not cytotoxic supporting its potential use in tissue engineering as a new, injectable, photocurable bone graft material.

Sun et al in 2006[15] designed a long-term study in which in vivo degradation of PCL was observed for 3 years in rats . Distribution, absorption and excretion of PCL were traced in rats using radioactive labeling. The results showed that PCL capsules with an initial Mw of 66,000 g/mol remained intact in shape during 2-year implantation, and broke into low Mw (8000 g/mol) pieces at the end of 30 months. The Mw of PCL deceased linearly with time. Tritium-labeled PCL (Mw 3000 g/mol) was subcutaneous implanted in rats to investigate its absorption and excretion and the radioactive tracer was first detected in plasma 15 days post-implantation. At the same time, radioactive excreta were recovered from feces and urine. An accumulative 92% of the implanted radioactive tracer was excreted from feces and urine at 135 days postimplantation. In parallel, the plasma radioactivity dropped to background level. Radioactivity in the organs was also close to background level, confirming that the material did not accumulate in body tissue and could be completely excreted which was in accordance with early studies.

Zhuang et al in 2009[16]have used *in situ* polymerization method to prepare polylactide (PLA)/ TiO2 nanocomposites with different contents of TiO_2. TiO_2 nanoparticles with a high aspect ratio possess many good properties such as

attractive photo catalytic activity, decent magnetic nature, fascinated hydrophilic and antibacterial properties. It was observed that when the amount of nano-TiO_2 is less than 3 wt.%, they dispersed throughout the PLA matrix in a quite uniform manner, and mechanical and thermal properties were found to be improved significantly .Composite degradation was better when the amount of TiO_2 increases, which was confirmed by UV light irradiation and solution degradation experiments .PLA/TiO_2 nanocomposites showed considerable bacteriostatic activity with the incorporation of TiO_2 nanoparticles in the nanocomposites.

Alani et al in 2009[17]aimed to develop a novel PCL/phosphate glass composite deliverable as a root filling and capable of releasing ionic species to enable a predictable seal in an aqueous environment. Different compositions of PCL-iron phosphate glass composites were produced and delivered into an ex vivo root canal model. Standardized root canals were prepared in extracted human teeth. The teeth were examined for root filling adaptation and precipitate formation (SEM), ion release (Na^+, Ca^{2+}, PO_4^{3-}, $P_2O_7^{4-}$, $P_3O_9^{3-}$, and $P_5O_{10}^{5-}$), and sealing ability. The experiments were controlled with teeth obturated with contemporary gutta-percha and a conventional zinc-oxide/eugenol sealer. The adaptation of the PCL composite was statistically significantly better than the control groups. Precipitate formation was noted in some specimens but all released various ionic species in an inverse proportion to the iron oxide concentration. The experimental material exhibited significantly less leakage after 7 days immersion in saline compared with those not immersed, or the control GP group. PCL-phosphate glass composites showed good potential as a root filling material capable of producing a seal in an aqueous environment without a sealer.

He .Y et al in 2009[18] concluded that Calcium sulfate (CaSO4*)*, as a commonly used implanting material, shows good biocompatibility, biodegradability, osteoconductivity and mechanical properties. Studies about using CaSO4 as bone filler for the treatment of bone defects are reported now and then, but the fabrication of injectable implant was hardly studied. In this study, calcium sulfate hemihydrate (CSH), as the basic material, was incorporated with a cellulose derivative, poly(ethylene glycol) (PEG), calcium sulfate dehydrate (CSD) crystal coated with PEG (P-CSD), and a certain amount of water to form injectable CaSO4 bone fillers. The structure of the bone fillers with different compositions was analyzed with scanning electron microscopy (SEM), infrared spectroscopy (IR) and X-ray diffraction (XRD). The effects of additives such as P-CSD, CSD, PEG and cellulose derivative on setting time, water absorption ability, mechanical properties and structure of the injectable bone fillers were studied. The *in vitro* degradation test showed that the injectable bone fillers have appropriate degradation time in phosphate-buffered solution (PBS), and they can maintain integrity throughout the degradation process. *In vitro* cell culture and preliminary animal model experiments demonstrated that the bone fillers do not exhibit a deleterious effect on cell viability and can hasten bone growth in bone defect model.

Su et al in 2010[19] have prepared biodegradable polycaprolactone / poly (glutamic acid) (PCL/PGA) blends by a melt blending method. Additionally, acrylic acid- grafted polycaprolactone (PCL-g-AA) was studied as an alternative to polycaprolactone. The samples were characterized using Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), an Instron mechanical tester, and scanning electron microscopy

(SEM). Because of poor compatibility between polycaprolactone and PGA, the mechanical properties of PCL/PGA blends were worse than of polycaprolactone alone. The PCL-g-AA/ PGA blends had obviously improved mechanical properties over PCL/PGA ones, and the former provided a plateau tensile strength at break when the PGA content was up to 20wt%. Biodegradation tests of blends were also conducted in a soil environment; the results showed that the mass of blends declined by about the PGA content within 4 weeks.

Parvin S et al in 2010[20] conducted a study based on quadruple hydrogen-bonding ureido-pyrimidinone (UPy) moieties hold promise as dynamic/stimuli-responsive materials in applications such as tissue engineering. Here, a new class of materials is introduced: supramolecular polymer composites. This study showed that despite the highly ordered structure and tacticity dependent nature of hydrogen-bonded supramolecular polymers, the bioactivity of these polymers can be tuned through composite preparation with bioceramics. The compression moduli increased about 40 and 90% upon composite preparation with HAp and HApUPy, respectively, as an indication to improved mechanical properties. These new materials show excellent potential as microporous composite scaffolds for the adhesion and proliferation of rat mesenchymal stem cells (rMSCs) as a first step toward bone regeneration studies; rMSCs proliferate about 2 and 2.7 times faster on the conventional composite with HAp and the supramolecular composite with (HApUPy) than on the neat PCL1250$(UPy)_2$.

Liu et al in 2010[13] studied the effect of nano-$CaCO_3$ on thermal and mechanical properties of PCL matrix. Incorporation of nano-$CaCO_3$ caused the

improvement in mechanical properties of the polycaprolactone matrix, whereas nano-$CaCO_3$ acted as a crystallization nucleating agent. Chemical foaming method was applied to prepare PCL/$CaCO_3$nano-composite. Parameters in relation with cell-like mean cell density, cell size, and cell wall thickness were analyzed. With different concentrations of $CaCO_3$ loading, variation in structural arrangement of cells of nano-composite foams has been observed. Cell wall thickness increased by an increase in $CaCO_3$ concentration and mean cell size achieved the minimum value at 5 wt.% concentration of $CaCO_3$. Mechanical properties of PCL/$CaCO_3$ nanocomposite foams were also optimized by changing the cell structure. With increasing $CaCO_3$ content, compressive strength of PCL/$CaCO_3$ nano-composite foams with similar density were increased too.

Liu JY et al in 2011[9] stated that Polycaprolactone (PCL)/calcium sulfate (CS) whisker composites have been fabricated by melt blending and coprecipitation methods respectively. Scanning electron microscope (SEM) was used to observe the microstructure of the composites. The crystallization and thermal properties were characterized by polarized optical microscope (POM), X-ray diffractometry (XRD), differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA). For composites prepared by melt blending method, experiment results show that average length of the whiskers is shortened. The crystallization perfection of PCL in composites is improved by adding whiskers. The flexural strength increases whereas the impact strength decreases. For composites prepared by coprecipitation method, whisker addition worsens the crystallization perfection of PCL. An improvement of 21% in flexural strength and 22% in impact strength has been achieved for the composite with 15 wt% of whiskers.

Bo Lei et al in 2012[21] examined the utility of sol–gel-derived bioactive glass microspheres (BGMs) as a reinforcement to improve the mechanical properties and biological performance of poly(e-caprolactone) (PCL) polymer. All of the PCLBGMs composites produced, with a variety of BGMs contents (10, 20, and 30 wt %), showed a uniform distribution of the BGMs in the PCL matrix, particularly owing to their spherical shape and small size. The PCL-BGMs composite with a BGMs content of 30 wt % showed vigorous growth of apatite crystals with a high aspect ratio on its surface after soaking in the simulated body fluid for 7 days, resulting in the creation of a porous carbonate hydroxyapatite layer.

Nekhamanurak et al in 2012[22] studied the effect of addition of $CaCO_3$ nanoparticles over the fracture behaviour and mechanical properties of PLA. Since synthetic silica possesses the key properties such as towering specific surface area and eminent absorbability that hinder the formation of firm linkage at the interface of two materials, initially the surfaces of $CaCO_3$ nanoparticles were made hydrophilic with silica coating by Sol-gel method. It was found that as the SiO_2 content on nanofiller increased, reinforcing effect of nano-$CaCO_3$ caused a simultaneous increase in elastic modulus, elongation at break and notched impact strength, proving the superior interaction of nanoparticles with PLA matrix after coating in nanocomposites.

Basilissi et al in 2013[23] prepared the PLA-silica nanocomposites by bulk Ring Opening Polymerization method (ROP) after treating nanosilica by two different oraganosilane agents. This treatment is necessary because of non-compatibility and poor adhesion between silica particles with PLA matrix that leads

to aggregation of mineral particles and deterioration of material properties. Highly loaded silane coated silica samples showed increased crystallization properties of nanocomposites. Thermal studies revealed that the thermal stability of PLA/silica nanocomposites was found to be much better compared to the neat PLA and made the nanocomposite compatible for processing.

Mu-noz-Bonilla et al in 2013[24] have prepared PCL/TiO_2nanocomposites by melt processing technique. They incorporated TiO_2 nanoparticles, synthesized by microemulsion route, with a mean diameter of about 10 nm, in concentrations ranging from 0.5 to 5 wt.%, into a biodegradable PCL matrix. A very good uniform dispersion of the oxide component of nanoparticle was achieved in the polymeric matrix, along with some aggregated submicrometer sized moieties. Anatase form of TiO_2 forms energy-rich electron-hole pairs upon UV excitation and these charge carriers are able to interact with microorganisms, executing their inherent bactericidal effect, even at nanometer scale. Through the analysis of the antimicrobial and/or optical properties, the bactericidal behavior of nanocompositesagainst Gram-positive Staphylococcus aureus and Gram-negative Escherichia coli has been explained with respect to increase in loading of nano-TiO_2 content.

Hassan MM et al in 2014[25] conducted a study to produce continuous fibers from different types of polymer at the nanometer scale by electrospinning technique. This study was conducted to produce tissue engineering scaffolds from Polycaprolactone (PCL) polymer solution by electrospinning technique and analyze its microscopic appearance to determine the morphology of nanofibers (scaffold).

The effect of ethanol on fiber morphology is also observed, as the morphology of fiber is supportive for cell adhesion. It has been observed that the maximum number of fibers have been produced with the diameter of 200 to 300 nm and the produced scaffolds are not significantly affected by ethanol.

Bajsic .EG et al in 2014[26] conducted a study based on 2.0 wt % of titanium dioxide (TiO_2) micro and nanoparticles were prepared by melt mixing and the effect of filler type and contents on the thermal properties, dynamic-mechanical behaviour and morphology were investigated. Measurements of storage modulus and loss modulus by dynamic mechanical analysis (DMA) showed better results for microfilled PCL/TiO_2 composites than nanofilled composites, with the same filler content. The crystallinity of the PCL increased with the addition of TiO_2 micro and nanoparticles; however, the χc for the PCL was unchanged with micro TiO_2 content. The thermal stability of PCL/TiO_2 composites were characterized using thermogravimetric analysis (TGA). The initial weight loss (5 wt %) occurs at slightly higher temperature with micro and nano TiO_2 addition and with increasing TiO_2 content.

Sarabi Maneji S et al in 2014[27] presented that polycaprolactonenanofibres were processed by electrospinning using a 3:1 ratio of tetrahydrofuran to methanol as solvent. The solvent choice was motivated by the possibility of greener alternatives to the halogenated compounds most often used for electrospinning. This paper presents the morphologies and fiber diameters resulting from the electrospinning of polycaprolactone solutions at room temperature under various conditions. The material morphology was characterized using scanning electron

microscopy and a measuring software. The process was optimized for smaller fibers with a narrower fiber diameter distribution by studying parameters such as polymer concentration, applied voltage, the tip to collector distance, and the solution flow rate. The experimental results were modelled using terminal jet diameter theory used in several referenced work. The theory behind this model was used to analyse the experimental observations made in the current paper. Process parameters were optimal for a 20% polycaprolactone concentration spun at a flow rate of 0.5 mL/h, with a tip to collector distance of 15 cm and an applied voltage of 8 kV. Fibers spun under these conditions displayed diameters of 546 ± 173 nm.

Nathalie et al in 2015[28] studied to optimize the procedure for coating electrospun poly(e-caprolactone) (PCL) fibers with a calcium phosphate (CP) layer in order to improve their potential as bone tissue engineering scaffold. In particular, attention was paid to the reproducibility of the procedure, the morphology of the coating, and the preservation of the porous structure of the scaffold. Ethanol dipping followed by an ultrasonic assisted hydrolysis of the fiber surface with sodium hydroxide solution efficiently activated the surface. Analysis of the cell viability, adhesion, and proliferation of MC_3T_3-E1 cells on untreated and CDHApcoated PCL scaffolds showed that the CDHAp coating enhanced the cell response, as the number of attached cells was higher in comparison to the untreated PCL and cells on the CDHAp coated samples showed similar morphologies as the ones found in the positive control.

Kolbuk D et al in 2016[5] stated that the effect of electrospinning parameters on morphology, molecular, and supermolecular structure of polycaprolactone (PCL)

fibers was analyzed, with respect to tissue engineering applications. Fibers morphology and structure are mainly determined by solution concentration and collector type. Applied voltage does not significantly influence supermolecular structure (crystallinity) and mechanical stiffness. There is correlation between changes in structure and proliferation of 3T3 cells as evidenced by in vitro study. Processing window of optimal scaffolds is relatively wide, however, variation of electrospinning parameters do not significantly affect their biological functionality.

MATERIALS & METHODS

The study protocol was approved by Institutional Research Committee (Reference No:- 09/07/2016) and Institutional Ethical Committee (Reference No: 12/2016) at Sree Mookambika Institute of Dental Sciences, Tamilnadu. Laboratory facilities for the study was provided by Biomedical Technology wing and Tissue Culture wing of Sree Chitra Thirunal Institute of medical sciences and research ,Trivandrum , India , for a period of 6 months.

MATERIALS USED

- Polycaprolactone (PCL) pellets with M_{WT} 80,000 (sigma Aldrich Pt Ltd, USA - [CP 1]
- Bioactive Calcium sulfate – [CP 2]
- Dimethylsulfoxide DMSO (C_2H_6OS),minimum 99% were purchased from spectrochem PVT.LTD, Mumbai,India. - [CP 3]
- Tetrahydrofuran (THF – packed under nitrogen C_4H_8O, minimum 99.9%) from spectrochem PVT.LTD, Mumbai, India - [CP 4]
- PCL incorporated with bioactive CaS – [CP 5]
- Electrospinning unit (Holmarc-USA) -Division of Tissue Culture, BMT wing, sree chitra tirunal institute of medical sciences and technology - [CP 6] Syringe Pump- [CP7]
- Electronic weighing machine – [CP 8]
- Thickness gauge – [CP 9]
- Automatic vernier caliper – [CP 10]
- FTIR Spectrometer – [CP11]

- Specimen prepared for SEM analysis – [CP12]
- Data physics OCA 15Plus for suface wettability – [CP 13]
- Homogenizer (Polytron –PT 1600E) – [CP14]
- Universal testing machine (Instron 3345,single column, UK) – [CP 15]
- Specimen prepared for direct contact analysis – (CP 16)
- Specimen prepared for cell adhesion analysis – (CP 17)
- Phase contrast microscope – (CP 18)
- UV irradiator for sterilization of samples
- L929 Mouse fibroblast cells
- Minimum Essential Medium (MEM, Sigma) supplemented with Foetal Bovine Serum(Gibco)
- CO_2 Incubator (Sanyo)

METHODS

Preparation of Polymer Solution

Membranes were fabricated by electrospinning PCL (10wt%) [CP-1] mixed in THF [CP-4] : DMSO [CP-3] at a ratio of 9:1 with different weight concentrations of calcium sulfate (5% and 10%).

Preparation of Polycaprolactone Solution

Solvent was prepared by mixing 10ml of THF & DMSO at a ratio of 9:1.0.1gm of PCL was weighed by electronic weighing machine [CP-8] & transferred to a 100ml glass beaker containing magnetic stir bar.10ml of solvent was added & dissolved PCL by continuous stirring on magnetic bar.

Polycaprolactone + Calcium Sulfate Solution (5%)

Bioactive Calcium sulfate powder [CP-2] was obtained from Biomedical technology (BMT) lab. Solvent was prepared as dissolved before. 0.5gm (5%) CaS dispersion was prepared by mixing(0.5gm CaS into 10ml of solvent). An even dispersion was obtained using a homogenizer (Polytron) – PT1600E [CP-14]. After 4hrs of stirring , a whitish homogenous solution was obtained.

Polycaprolactone + Calcium Sulfate Solution (10%)

Bioactive Calcium sulfate powder was obtained from Biomedical technology (BMT) lab. Solvent was prepared as dissolved before. 1gm (10%) CaS dispersion was prepared by mixing (1gm CaS into 10ml of solvent). An even dispersion was obtained using a homogenizer (Polytron) – PT1600E. After 4hrs of stirring, a whitish homogenous solution was obtained.

Electrospinning Setup for GTR membrane fabrication

The PCL and PCL-CaS solutions were electrospun in a uniform spinning configuration, using a customized electrospinning unit (Holmarc, USA) [CP-6]. Polymer solutions were supplied continuously to a syringe connected to a 21 gauge blunt end needle with a feeding rate [CP-7] of 1.5 ml/h for 4hr. A high voltage of 12 kV was applied between the needle kept at a distance of 16 cm from rotating mandrel set at 200 revolutions per minute. The fibrous mats were detached from stationary mandrel, rinsed twice in deionized water to remove the solvent and air dried at 37^{0}C overnight. The scaffolds were sterilized by exposing to UV for 30 min prior to cell culture studies.

CELL CULTURE MATERIALS AND METHODS

Materials and Methods used for Direct Contact

- Source of cell line - ATCC - Strain L-929
- Justification - L-929 is an established and well characterised mammalian cellline that has demonstrated reproducible results.
- Culture Medium - Minimum Essential Medium supplemented with Foetal Bovine Serum

Test details

Assay Method and Rationale	-	Direct Contact (ISO 10993-5, 2009) (As requested by the customer)
Test sample preparation	-	Samples were sterilized by UV by overnight (If applicable)
Negative control	-	Ultra High Molecular Weight Poly Ethylene
Preparation of negative control	-	Not applicable
Positive control	-	Stabilized PVC Disc
Preparation of positive control	-	Not applicable

Test procedure

An in vitro cytotoxicity test using Direct contact method was performed using test samples PCL and PCL CAS, as per ISO 10993-5. The culture medium from the L-929 monolayer was replaced with fresh medium. Test samples, negative controls and positive controls in triplicate were placed on the cells. After incubation at 37± 1°C for 24 to 26h, cell monolayer was examined microscopically for the response around the test samples. The reactivity were graded as 0, 1,2, 3 and 4 based on zone of lysis, vacuolization, detachment and membrane disintegration as per the table given below.

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild Zone	limited to area under specimen
3	Moderate Zone	extending specimen size up to 0.33 cm
4	Severe Zone	extending farther than 0.33 cm beyond specimen

Materials and Methods for Cell adhesion

Cell Culture

Source of cell line - ATCC - Strain L-929

Justification - L-929 is an established and well characterised mammalian cell line that has demonstrated reproducible results.

Culture Medium - Minimum Essential Medium supplemented with Foetal Bovine Serum

Test details

Assay Method and Rationale - Cell adhesion study
(As requested by the customer)

Test sample preparation - Samples were sterilized by UV by overnight
(If applicable)

Control - Glass cover slip

Test procedure

An in vitro cell adhesion test was performed with test materials PCL and PCL CaS. L929cells were trypsinised, seeded on test materials and control glass

cover slip at density of 1×104 cells/cm^2 and incubated for 48 h at 37±1ºC under humidified atmosphere containing 5% CO2. After 48h, cell seeded test materials and glass cover slips were rinsed with PBS and fixed in 4% para-formaldehyde. Fixed samples were rinsed thrice with phosphate buffered saline (1X PBS) and permeabilised by treating with 0.1% Triton-X100 for 1 min. The samples were rinsed with PBS and incubated with rhodamin conjugated phalloidin for 15min. Cell nuclei was counter stained with Hoechst 33258. The cell adhesion and morphology was observed under fluorescence microscope (Leica DMI 6000B).

CHARACTERIZATION OF ELECTROSPUN SCAFFOLDS

Mechanical and chemical properties of scaffolds were characterized using scanning electron microscopy (SEM), Fourier transform infra-red spectroscopy (FTIR), EDAX and Universal testing machine.

Morphology Analysis

The three dimensional(3D) morphological (fiber diameter and pore diameter, fiber distribution and pore distribution) and structural aspects of electrospun GTR mats was observed by scanning electron microscope (Hitachi –model-2400). In order to study the fiber morphology, the PCL and PCL-Calcium sulfate samples were coated with gold in a sputter coating device and observed under SEM.

The digital images from selected areas were transferred to Image J software to analyze the fiber diameter and pore diameter. The fiber size distribution measured by image analysis of PCL and PCL-CaS showed variations in the fiber diameter in the range 0.05–0.23 microns.

Elemental Identification

The elemental identification of PCL and PCL-CaS electrospun fibrous mat was determined by using EDAX.

Tensile Strength

The mechanical characterization of PCL and PCL-CaS fibrous mats were evaluated by using Universal testing machine (Instron 3345,single column,UK)[CP-15]. Rectangular strips of 1 x 5cm PCL and PCL-CaS fibrous mats having thickness (measured by using thickness gauge [CP-9]) ranging from 0.05 – 0.23mm was used for analysis at a crosshead speed of 10mm/min. In order to avoid the reading error five set of electrospun fibrous mat samples each were tested to evaluate Tensile strength.

Suture Pullout Test

Suture retention strengths of the electrospun PCL and PCL–CaS mats (1div=0.01mm, 5 mm width, 0.25 mm thickness, and 7mm length) were measured using a mechanical tester (Instron 3345,single column,UK). One end of the specimen was fixed with the stage clamp of the tester, and the other end was connected to another clamp by a suture material (5-0 Prolene, Ethicon, Piscataway, NJ, USA). The suture was placed 2 mm from the end of the scaffold. The measurement was performed using a 2 kN maximum load cell with a cross-head speed of 8 mm/min. A tensile force was applied until the suture specimen was completely stretched off. The rupture stress was recorded.

Surface wettability of electrospun fibrous mat

The wettability of the electrospun fibrous mat of PCL and PCL-CaS were measured at room temperature using Data Physics OCA 15plus, Germany [CP-14]

by sessile drop method. Water drops of 5µl were placed at different areas in a glass slide of three samples each. Then the contact angle was recorded and image was taken.

Fourier transform infra-red spectroscopy (FTIR)

Chemical characterization of electrospun mats were analyzed by FTIR [CP-11]. Here the presence of sulfate (SO_4) group on PCL-CaS fibers was analyzed by using FTIR.

Cell Culture Studies

Direct Contact

In vitro cytotoxicity of PCL and PCL-CaS was performed by Direct contact method as per ISO 10993-5. L929 cells maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 10% Foetal Bovine Serum (Gibco) and Penicillin-Streptomycin antibiotic (Pen- Strep10,000U/ml) was enzymatically obtained (0.25 % Trypsin and 0.02% EDTA) in suspension. Approximately 3×10^4 cells were seeded to 24 well plates and allowed to form a monolayer at 37°C in a CO_2 incubator. Following the incubation, culture medium from the L-929 monolayer was replaced with fresh medium and the test samples, negative controls (Ultra high density polyethylene) and positive controls (Stabilised PVC disc) in triplicate were carefully placed on the cells. After 24h incubation, cell monolayer was examined microscopically for the response around the test samples.

The reactivity were graded based on zone of lysis, vacuolization, detachment and membrane disintegration using an inverted phase contrast microscope (Motic, USA) [CP 18].

Cell Adhesion

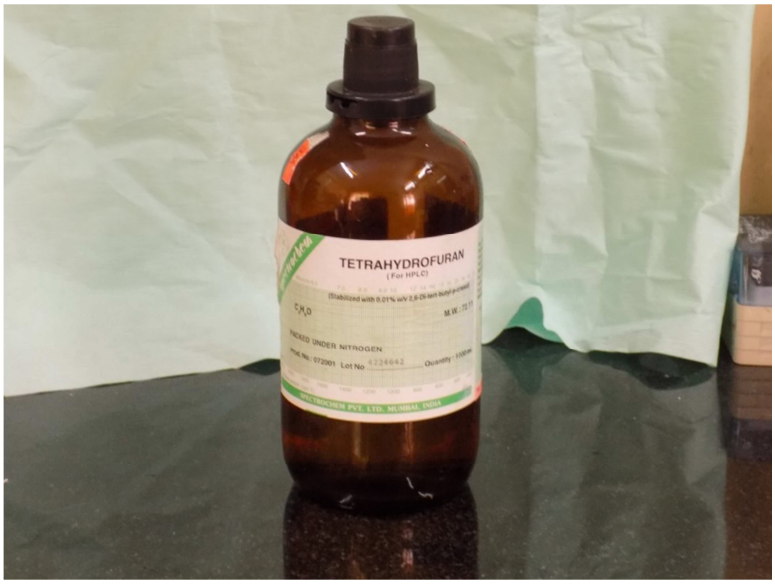
The cell adhesion on PCL and PCL-CaS was evaluated using L-929 cells. Approximately 1 x 10^4 cells were seeded onto the test materials and was incubated for 48 h at 37°C in a CO_2 incubator. Condition test material with sufficient volume of medium (Make sure to remove the condition medium before cell seeding). The morphology of adhered cells on materials were analyzed by staining actin filaments. Cells adhered on PCL mats were fixed using 4% paraformaldehyde (Sigma) for 1h. The cells were rinsed with PBS and permeabilized with 0.1% Triton-X100for 5min. After removing detergent, cells were rinsed and incubated with rhodamine conjugated with Phalloidin (50 μg/ml, 20 min)purchased from Life Technology. Cell nuclei were counter stained with Hoechst(0.5 μg/ml) for 1min. The morphology of the cells on material was observed under fluorescence microscope.

COLOR PLATES

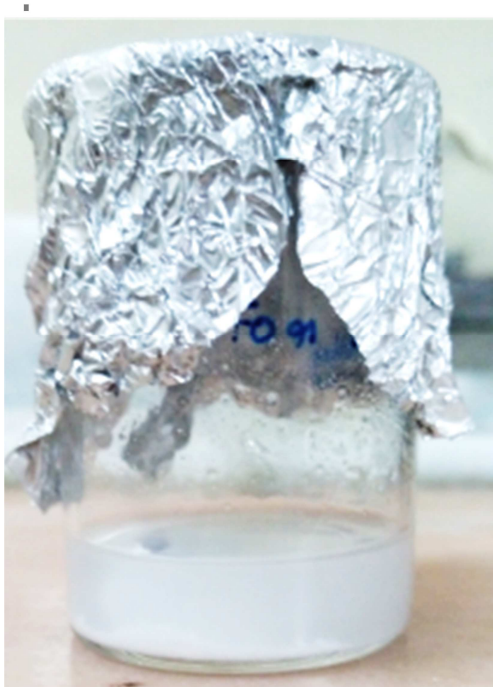
CP 1. Polycaprolactone Pellets

CP 2. Bioactive calcium sulfate

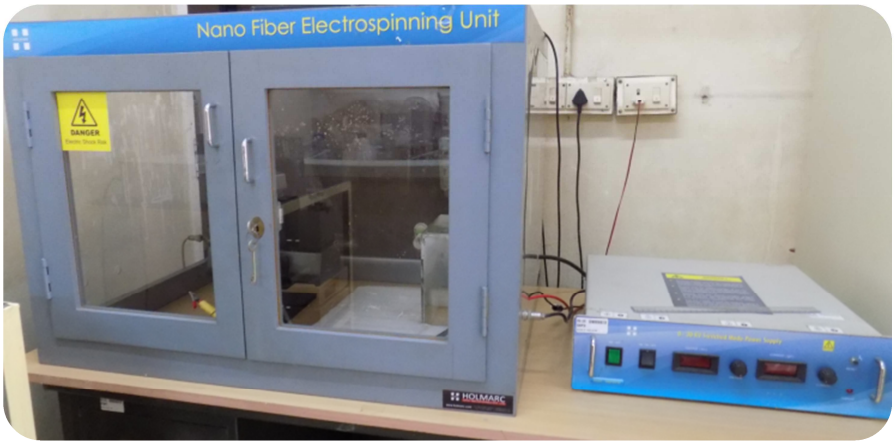
CP 3. Dimethyl sulfoxide anhydrous



CP 4. Tetrahydrofuran



CP 5. PCL incorporated with bioactive CaS solution



CP 6. Electrospinning unit

CP 7. Syringe pump

CP 8. Electronic weighing machine

CP 9. Thickness gauge

CP 10. Automatic Vernier Caliper

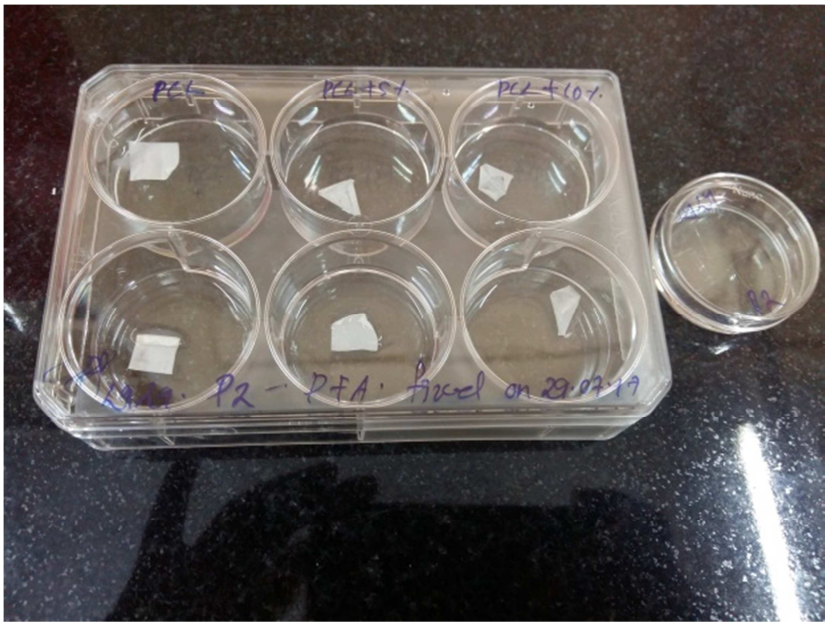
CP 11. FTIR Spectrometer

CP 12. Specimen prepared for SEM analysis

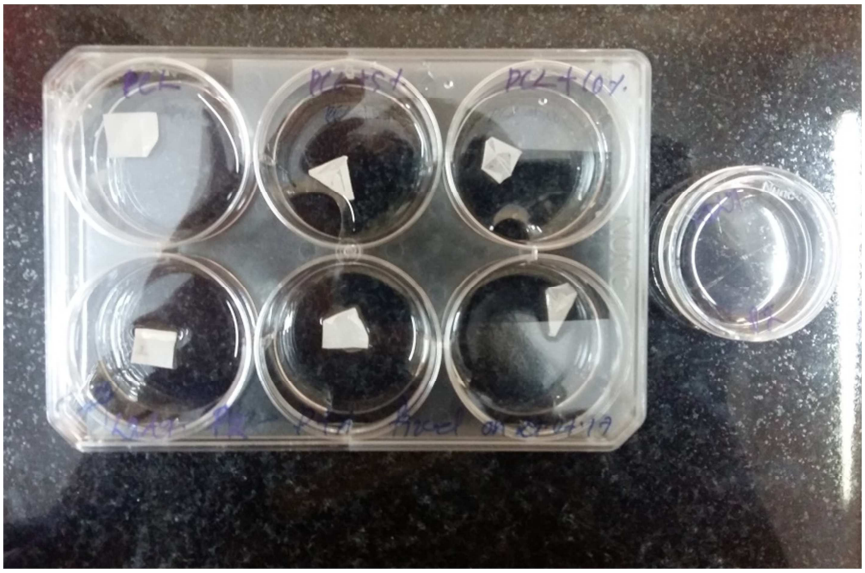
CP 13. Data physics OCA 15 Plus for surface wettability

CP 14. Homgenizer – Polytron –PT1600E

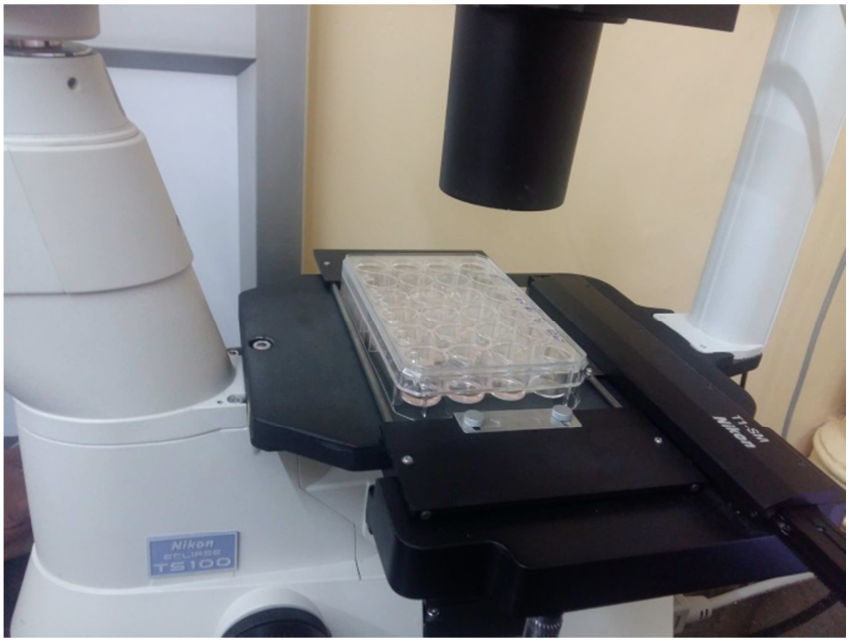
CP 15. Universal testing machine (Instron 3345, Single column, UK)



CP 16. Specimen Prepared For Direct Contact Analysis



CP 17. Specimen Prepared For Cell Adhesion



CP 18. Phase contrast microscope

RESULTS & OBSERVATIONS

Fabrication of electrospun fibrous mats

The PCL and PCL-CaS blend solutions were electrospun to get microfibrous mats. The effect of the blending concentration in fiber formation was determined at different concentrations of Calcium Sulfate with PCL.

Scanning electron microscopy

The PCL-CaS blend fibrous mat was compared with PCLmat. The PCL mats obtained from the THF: DMSO solvent showed very fine and dense fibers. The PCL-CaS fibrous mats at weight above 10% showed extensive bead formation (data not shown). The fibrous mats of 5% and 10% CaS and PCL weight ratio formed homogeneous fibers without bead formation. A marked reduction in fiber density and increase in fiber diameter was noted in PCL-CaS scaffolds. The fiber size distribution measured by image analysis of PCL and PCL-CaS showed variations in the fiber diameter in the range 0.05–0.23m.

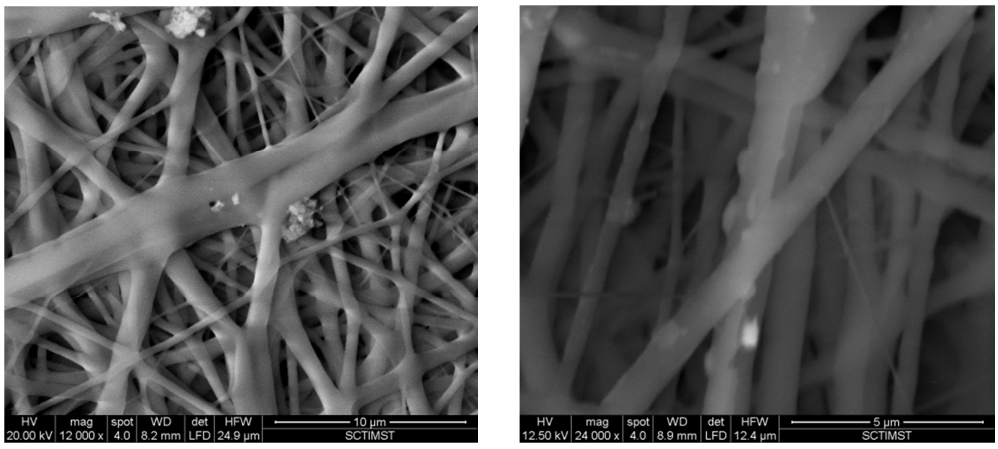


Image 1 & 2. SEM micrographs showing electrospun fibrous mats of PCL-CaS.

Tensile Strength

The mechanical characterization of PCL and PCL-CaS fibrous mats were evaluated by using Universal testing machine (Instron 3345,single column, UK).Results of tensile testing revealed that the tensile properties of PCL-CaS scaffolds improved by fiber bonding and distribution. The tensile strength of electrospun PCL scaffolds exhibited value is 5.564MPa and incorporation of 5%,10% calcium sulfate along with PCL exhibited as 3.122MPa* (5%) and 4.236MPa* (10%) respectively which shows appropriate value with PCL.

Suture Pull Out Test

The ability to withstand forces resulting from suturing was measured by suture retention strength. The suture retention strength of electrospun PCL scaffold is0.19 ± 0.23 and where calcium sulfate incorporated PCL exhibited value is 0.41±0.26(5%CaS) and 0.52±0.24 (10%CaS) respectively.

Surface wettability of electrospun fibrous mat

The PCL fibrous mat showed water contact angle of 130^0 ± 4.72, whereas 5% PCL-CaS blend fibrous mat expressed lower values of 128^0± 2 and 10% CS-PCL blend expressed 118^0 ±1.5. This confirms that PCL-CaS mats is relatively more hydrophilic compared to that of PCL.

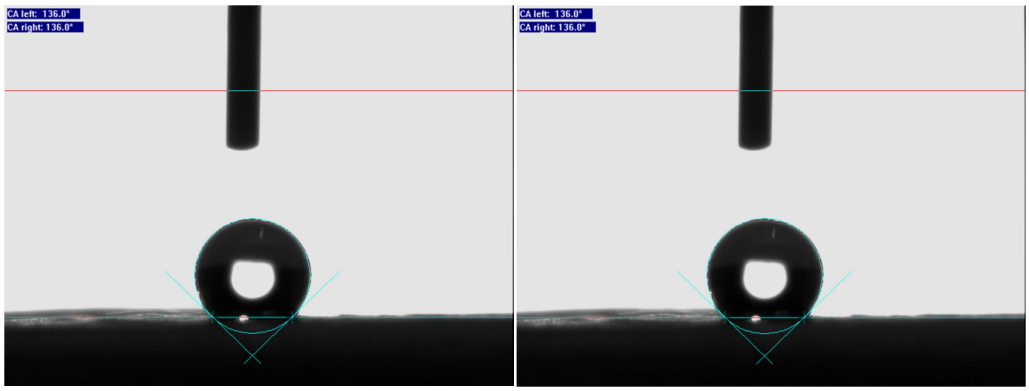


Image 3 & 4. The water contact angle of (a) PCL (130^0 ± 4.72) and (b) PCL-CaS (119^0.67±1.52)

Cell culture Studies

Direct Contact Assay with L929 mouse fibrobast

As per ISO 10993-5 the achievement of numerical grade more than 2 is considered as cytotoxic effect. Since the test material PCL, PCL-CaS achieved a numerical grade not greater than 2, the material is considered as not cytotoxic. . The reactivity were graded based on zone of lysis, vacuolization, detachment and membrane disintegration using an inverted phase contrast microscope (Motic, USA). Negative control gave none cytotoxic reactivity and positive control gave severe cytotoxic reactivity as expected.

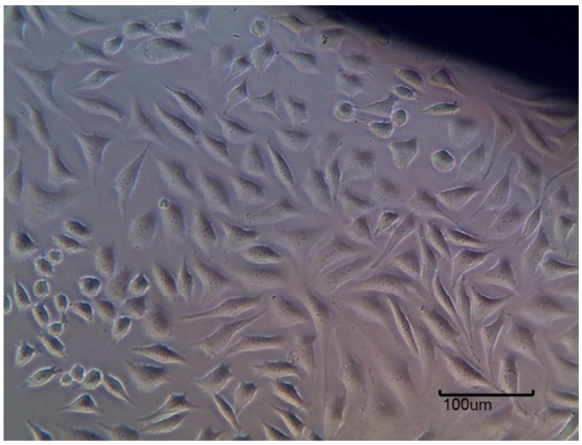


Image 5. Negative Control

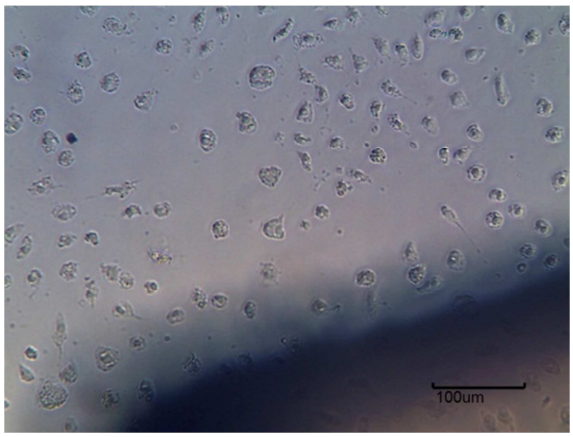


Image 6. Positive Control

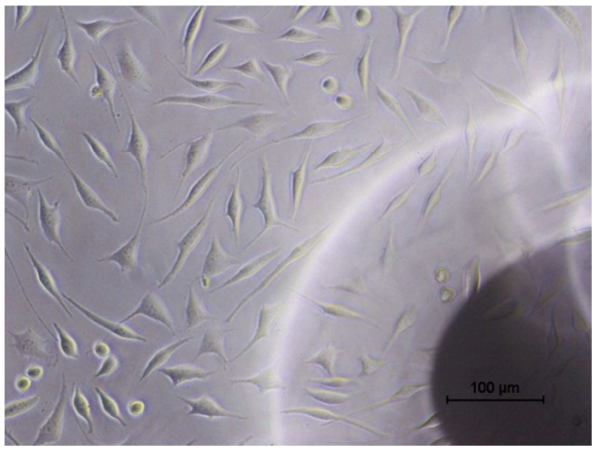


Image 7. PCL

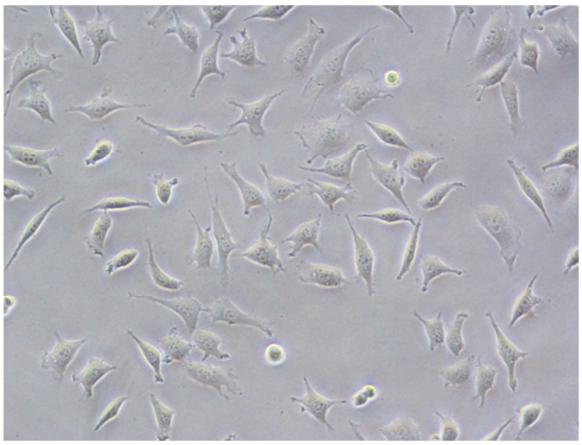


Image 8. PCL+5% CaS

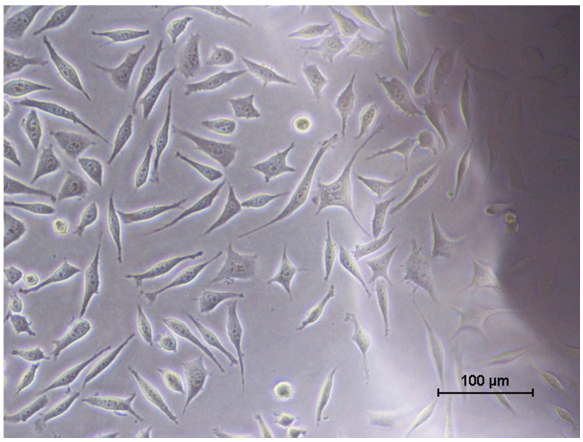
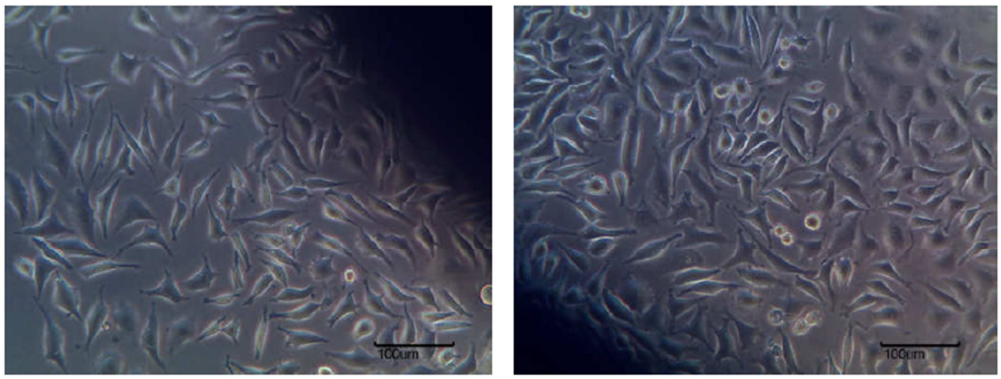


Image 9. PCL+10% CaS



a) L929 cells around PCL b) L929 cells around PCL CAS

Image 10 & 11. L929 cells around PCL and PCL-CaS

CELL ADHESION

Cells were rinsed and incubated with rhodamine conjugated with Phalloidin (50 μg/ml, 20 min) purchased from Life Technology. Cell nuclei were counter stained with Hoechst(0.5 μg/ml) for 1min. The morphology of the cells on material was observed under fluorescence microscope.L929 cells were adhered and well spread on test samples PCL and PCL-CaS. It shows an excellent cytoskeletal architecture.

Control

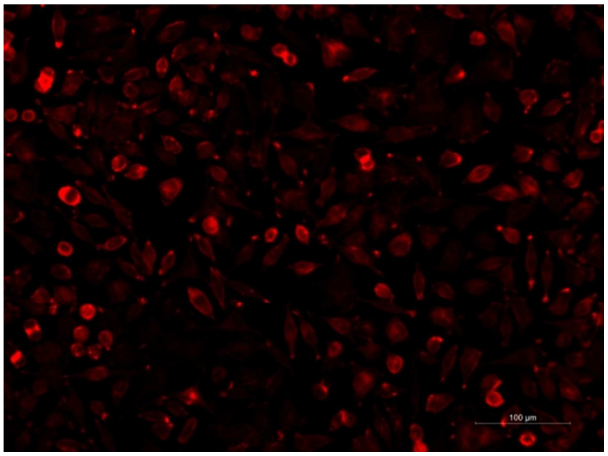


Image 12. Rhodamine conjugated phalloidin (PCL)

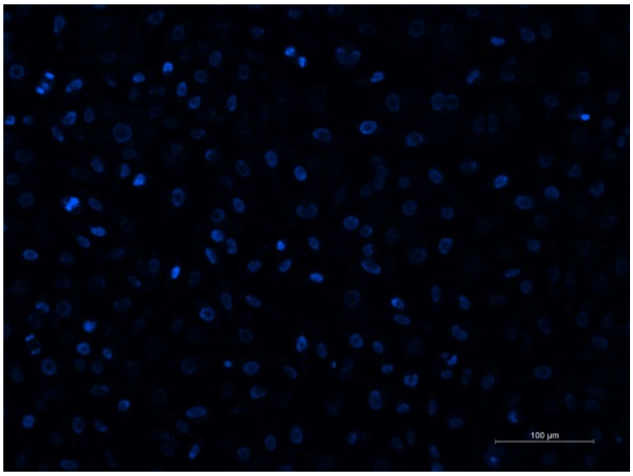


Image 13. Nucleus stained with Hoechst (PCL)

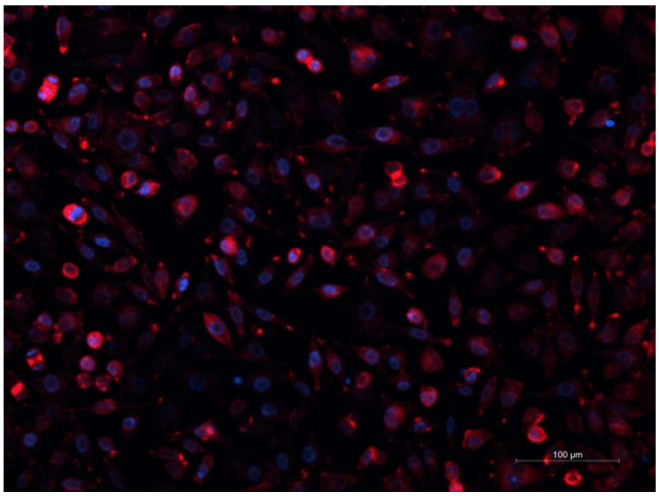


Image 14. Overlay(PCL)

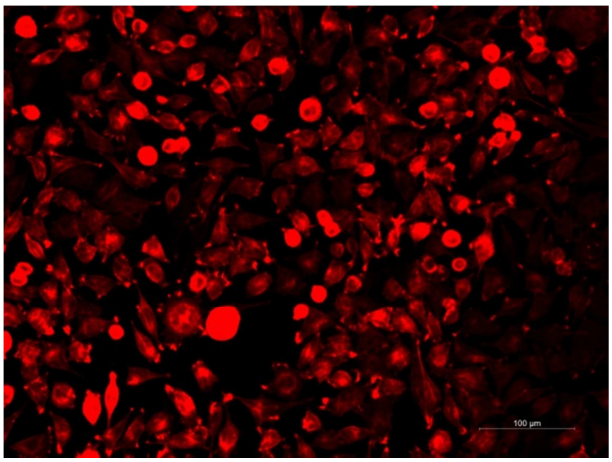


Image 15. Rhodamine conjugated phalloidin (PCL+5%CaS)

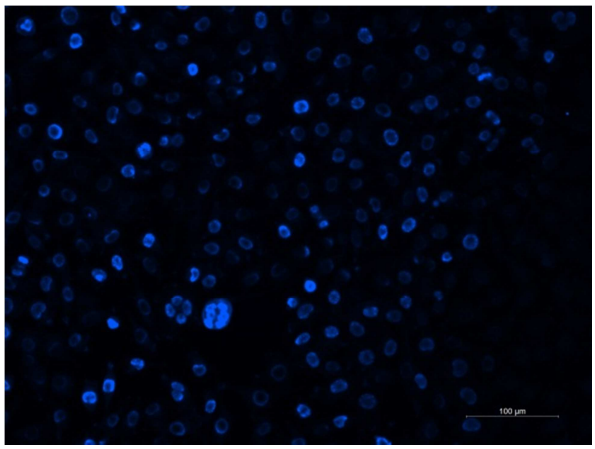


Image 16. Nucleus stained with Hoechst (PCL+5%CaS)

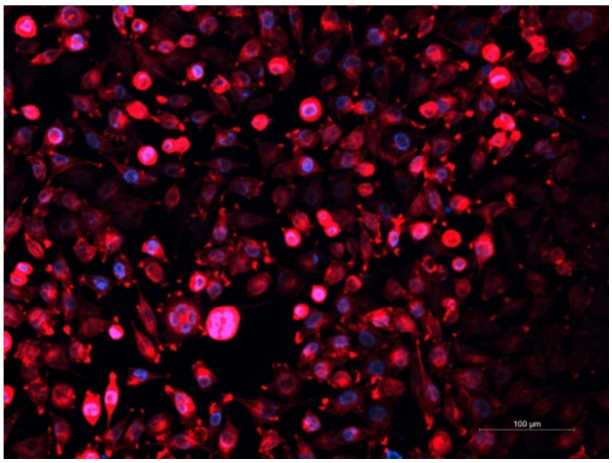


Image 17. Overlay (PCL+5%CaS)

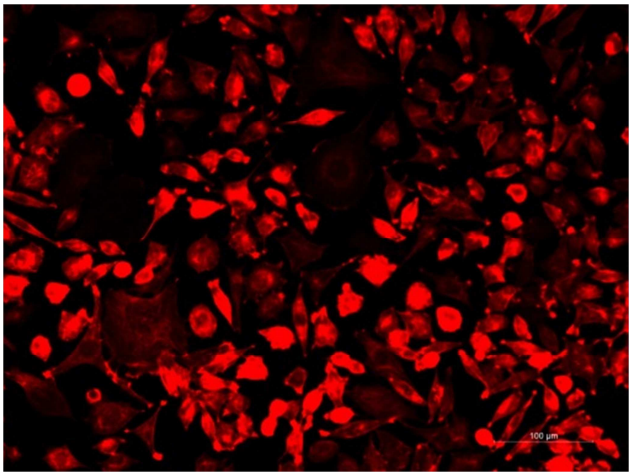


Image 18. Rhodamine conjugated phalloidin (PCL+10%CaS)

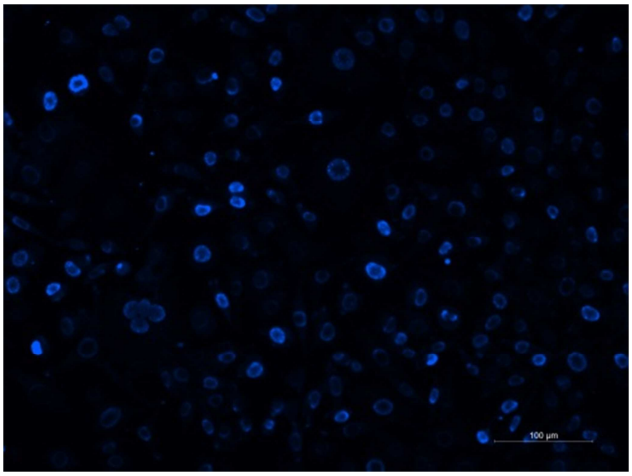


Image 19. Nucleus stained with Hoechst (PCL+10%CaS)

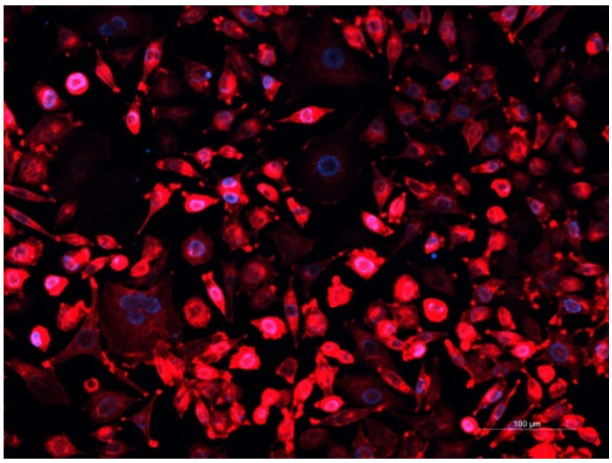


Image 20. Overlay (PCL+10%CaS)

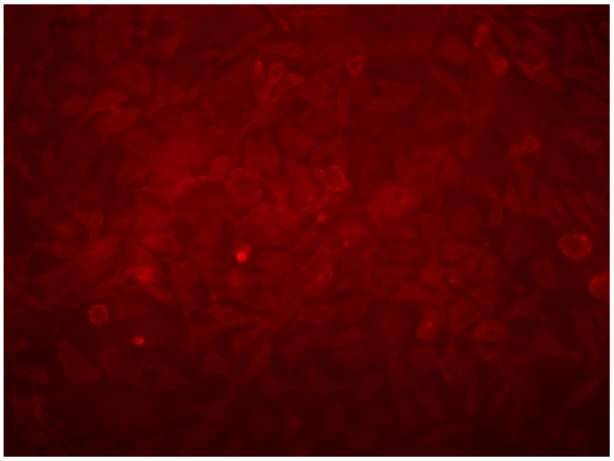


Image 21. Rhodamine conjugated phalloidin (PCL)

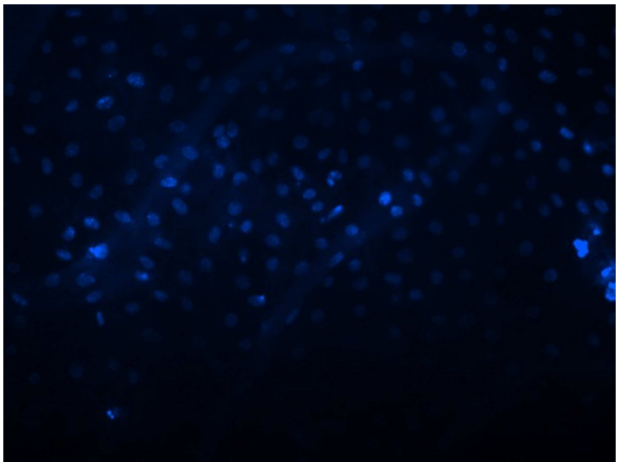


Image 22. Nucleus stained with Hoechst (PCL)

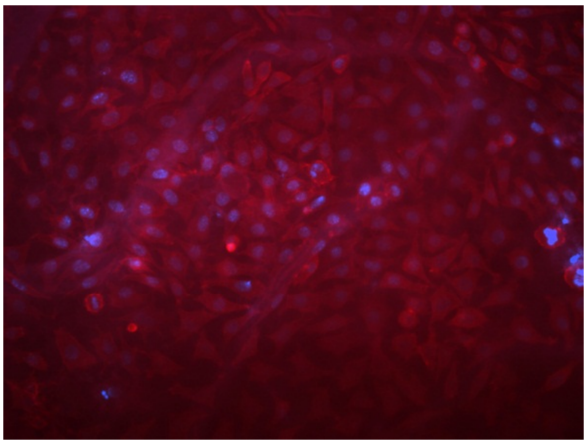


Image 23. Overlay(PCL)

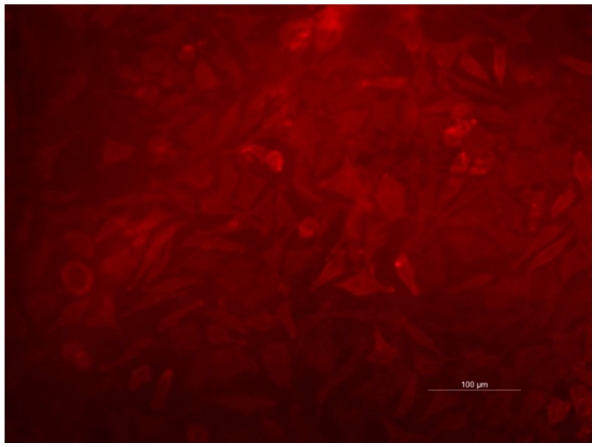


Image 24. Rhodamine conjugated phalloidin (PCL+5%CaS)

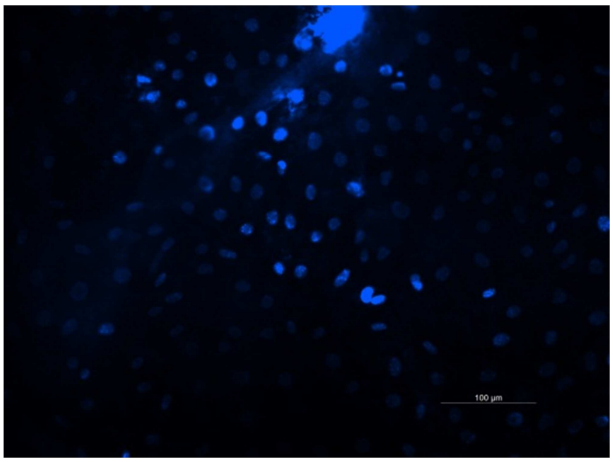


Image 25. Nucleus stained with Hoechst (PCL+5%CaS)

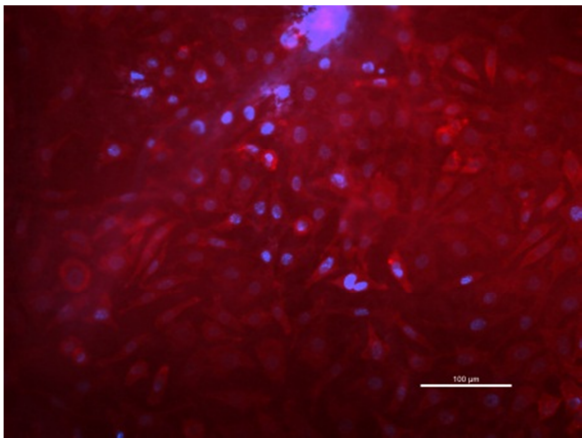


Image 26. Overlay (PCL+5%CaS)

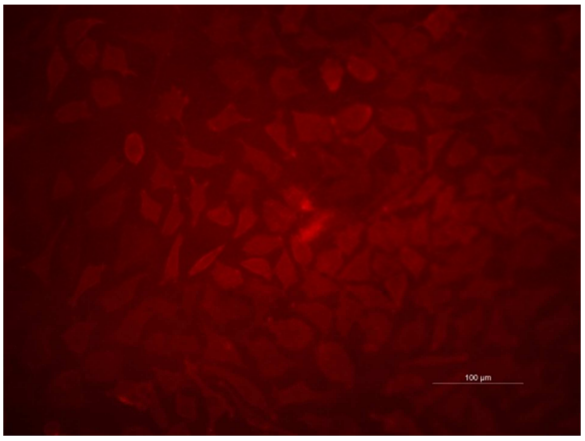


Image 27. Rhodamine conjugated phalloidin (PCL+10%CaS)

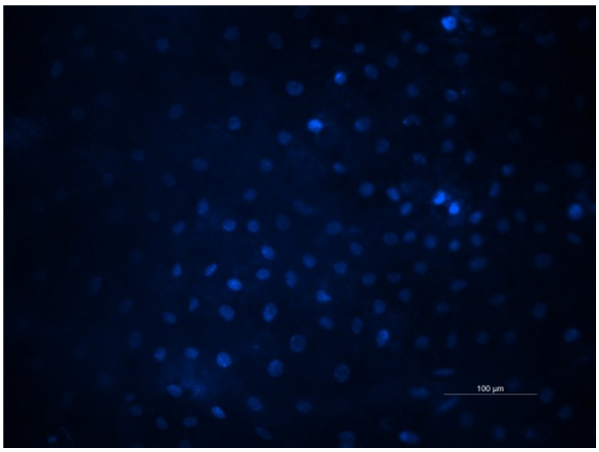


Image 28. Nucleus stained with Hoechst(PCL+10%CaS)

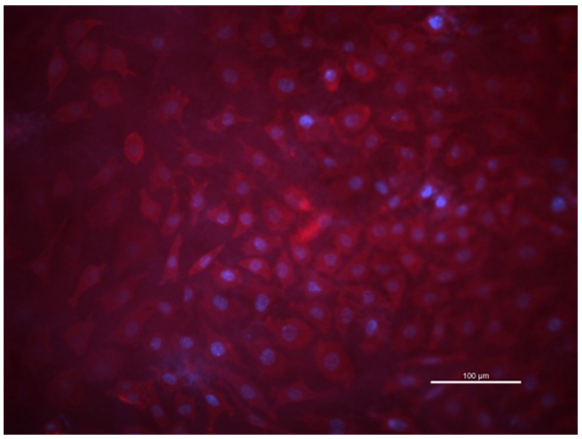
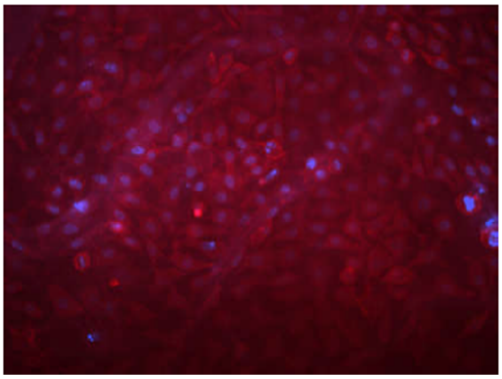


Image 29. Overlay (PCL+10%CaS)



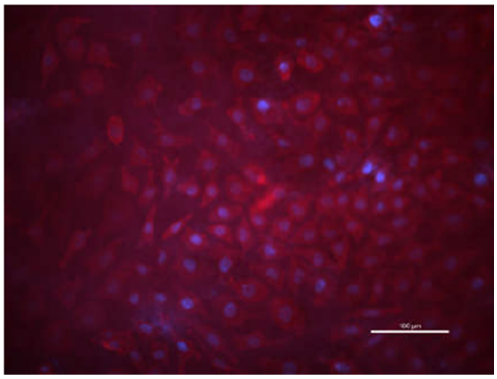


Image 30 & 31. L929 cells around PCL and PCL-CaS

Statistical Analysis

The data was expressed in mean and standard deviation. Statistical Package for Social Sciences (16.0) version used for analysis. ANOVA (Post hoc) followed by Dunnet t-test applied to find the statistical significant between the groups. P value less than 0.05 ($p<0.05$) considered statistically significant at 95% confidence interval.

Table-1: Mean tensile strength values of different groups

Groups	Treatment	Tensile strength (MPa) (MEAN±SD)
Group-I	**PCL**	5.56±0.70
Group-II	**PCL+5% CaS**	3.12±0.27
Group-III	**PCL+10% CaS**	4.23±0.61

Table 1.shows the mean tensile strength of different groups. The tensile strength of Group I (PCL) showed higher value compared to other two groups(PCL+5%CaS and PCL+10%CaS).

Table-2: Mean suture pull out values of different groups

Groups	Treatment	Suture pull out (N) (MEAN±SD)
Group-I	**PCL**	0.19±0.23
Group-II	**PCL+5% CaS**	0.42±0.29
Group-III	**PCL+10% CaS**	0.46±0.24

Table 2, shows the mean suture pull out of different groups. Group I exhibited the value as 0.19±0.23, group II exhibited 0.42±0.29 and Group III exhibited 0.46 ±0.24. The suture pull out of Group III (PCL+10% CaS) showed higher value compared to other two groups(PCL and PCL+5%CaS).

Table-3: Mean water contact angle of different groups

Groups	Treatment	Water contact angle (^{0}) (MEAN±SD)
Group-I	**PCL**	130.67±4.72
Group-II	**PCL+5% CaS**	128.00±2.00
Group-III	**PCL+10% CaS**	119.67±1.52

Table 3, shows the mean water contact angle of different groups. The water contact angle of Group III (PCL+10% CaS) showed lower value compared to other two groups(PCL and PCL+5%CaS).

Table-4: Multiple comparisons of mean tensile strength values between the groups

Groups	Treatment	Tensile strength (MPa) (MEAN±SD)	p value
Group-I	**PCL**	5.56±0.70	0.03
Group-II	**PCL+5% CaS**	3.12±0.27*	0.04
Group-III	**PCL+10% CaS**	4.23±0.61*,#	0.03

(*$p<0.05$ significant compared Group-I with other groups, #$p<0.05$ significant compared Group-II with other groups)

Table 4 shows multiple comparison of tensile strength between the three groups. Statistically significant higher tensile strength was exhibited by the PCL membrane [5.56±0.70, $p<0.05$] when compared with other two groups [PCL+5%CaS and PCL+10%CaS]. The data exhibited by the other two membranes [PCL+5%CaS and PCL+10%CaS] was also statistically significant.

Table-5: Multiple comparison of mean suture pull out values between the groups

Groups	Treatment	Suture pull out (N) (MEAN±SD)	p value
Group-I	**PCL**	0.19±0.23	0.04
Group-II	**PCL+5% CaS**	0.42±0.29*	0.04
Group-III	**PCL+10% CaS**	0.46±0.24*	0.04

(*$p<0.05$ significant compared Group-I with other groups, $p>0.05$ no significant difference compared Group-II with other groups)

Table 5 shows multiple comparison of suture pull out test between the three groups. Statistically significant higher suture pull out data was exhibited by PCL+10%CaS membrane [0.46±0.24*, $p<0.05$] when compared with other two groups [PCL and PCL+5%CaS].

Table-6: Multiple comparison of mean water contact angle values between the groups

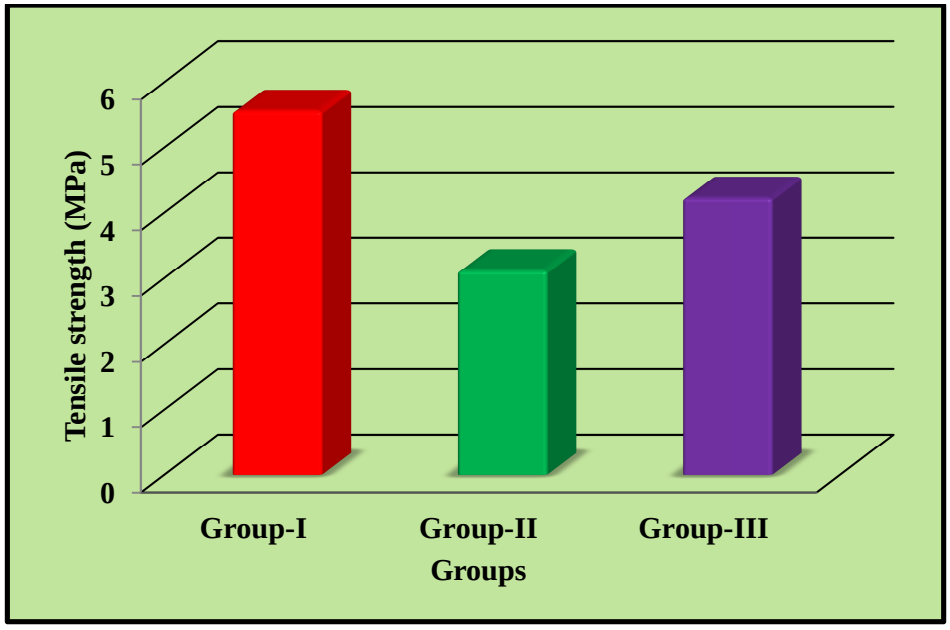
Groups	Treatment	Water contact angle (^{0}) (MEAN±SD)	p value
Group-I	**PCL**	130.67±4.72	0.05
Group-II	**PCL+5% CaS**	128.00±2.00*	0.03
Group-III	**PCL+10% CaS**	119.67±1.52*,#	0.03

(*p<0.05 significant compared Group-I with other groups, #p<0.05 significant compared Group-II with other groups)

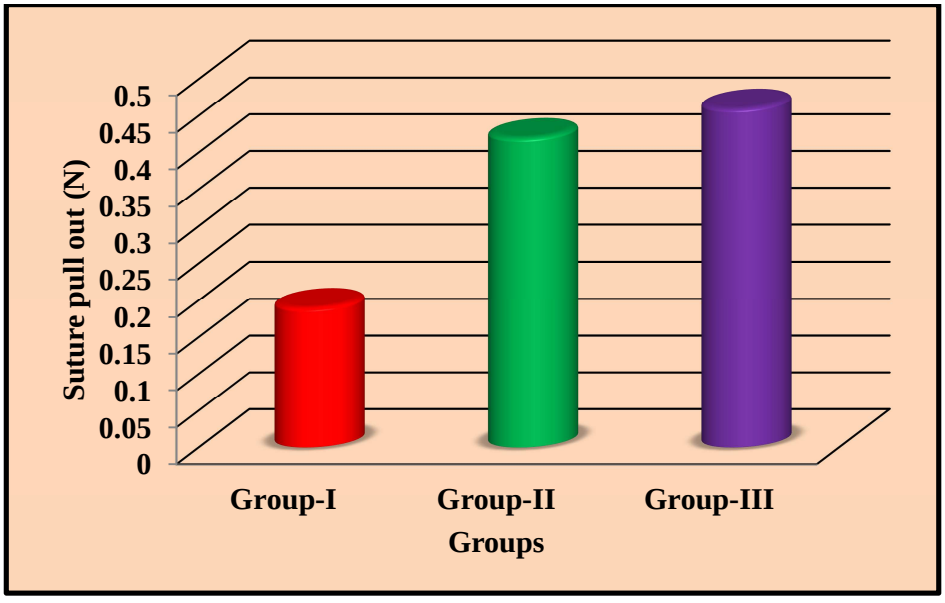
Table 6 shows multiple comparison of water contact angle values between the three groups. Statistically significant higher water contact angle data was exhibited by PCL+10%CaS membrane [119.67±1.52*,# p<0.05] when compared with other two groups [PCL and PCL+5%CaS].

GRAPHS

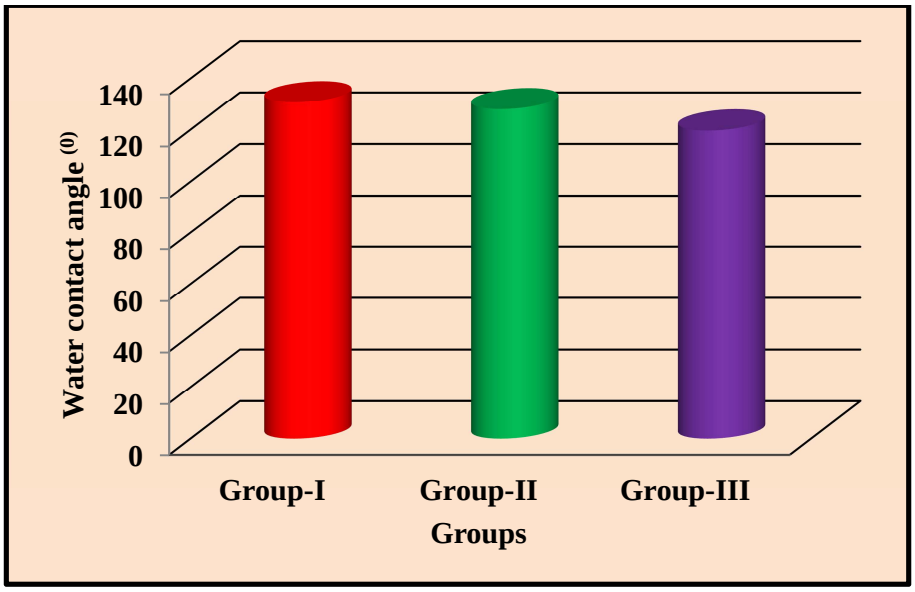
Graph-1: Multiple comparisons of mean tensile strength values between the groups



Graph-2: Multiple comparison of mean suture pull out values between the groups

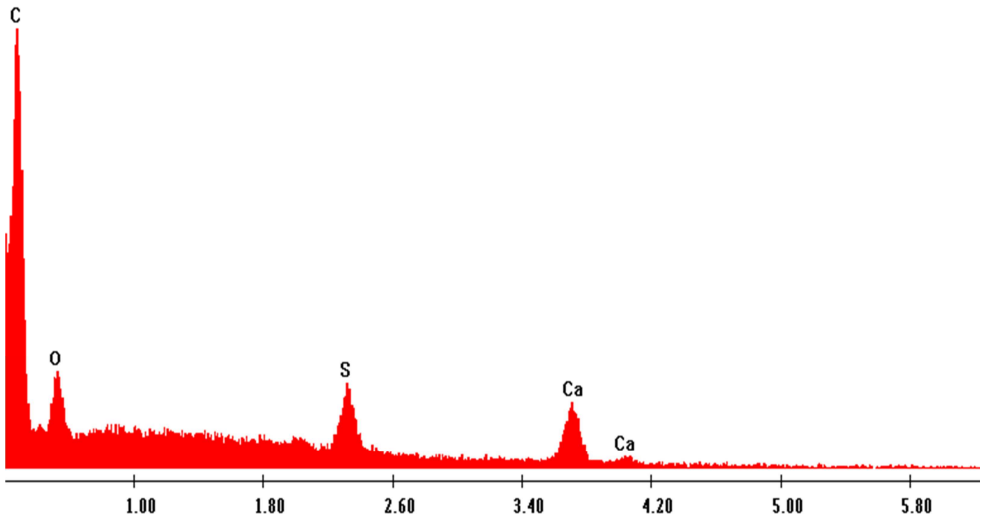


Graph-3: Multiple comparison of mean water contact angle values between the groups



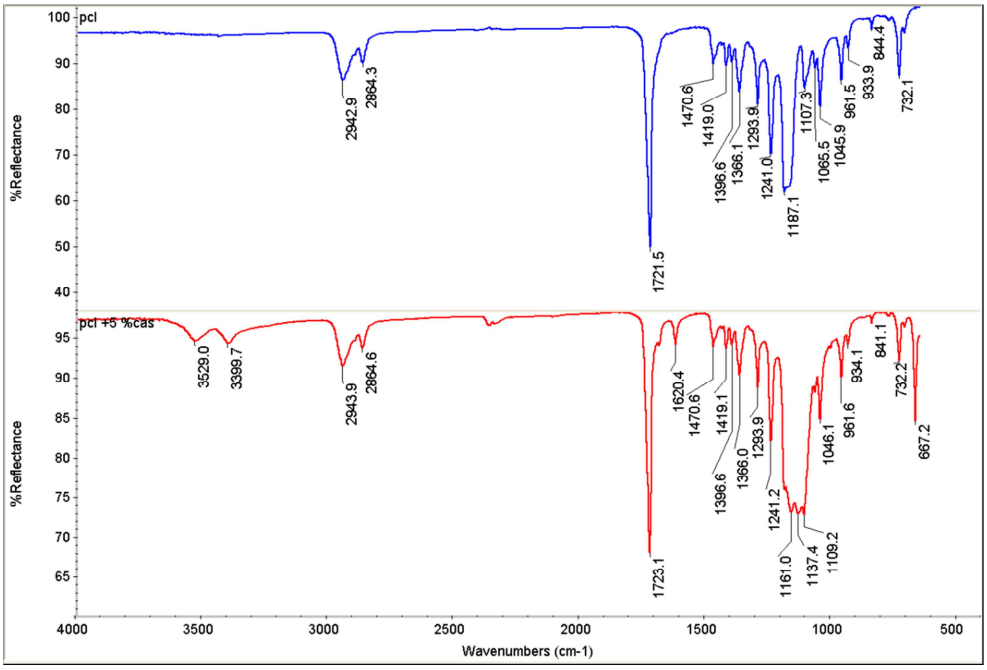
Elemental Identification

Graph 4. Elemental Identification of PCL-CaS electrospun fibrous mat.

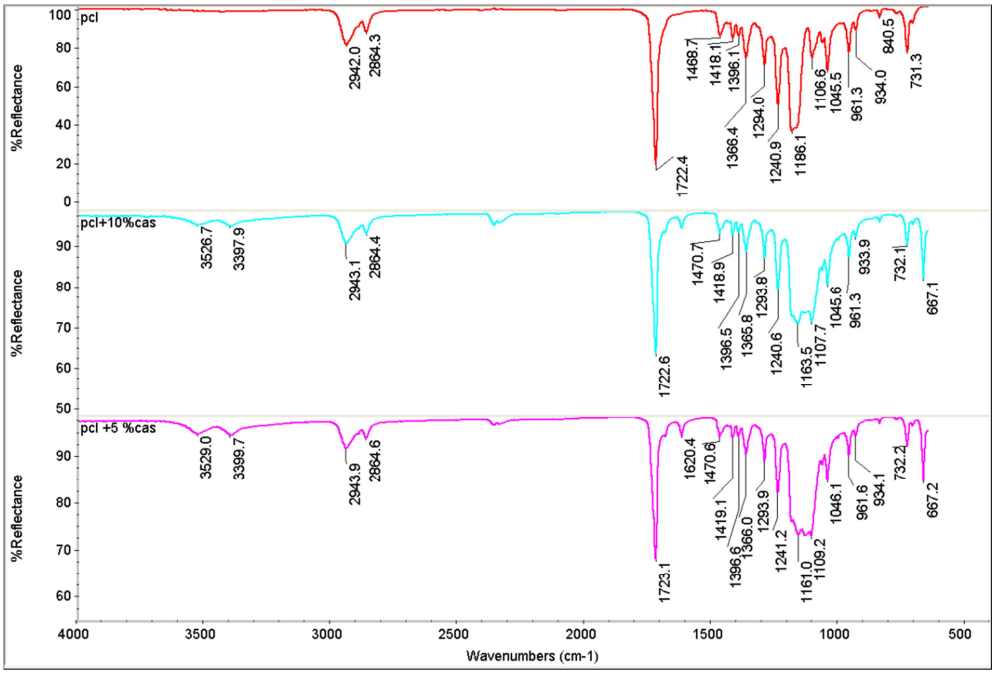


Spectroscopic Analysis

Graph 5. Spectral analysis of PCL and PCL+5%CaS



Graph 6. Spectral analysis of PCL , PCL+10%CaS and PCL+5%CaS



DISCUSSION

Electrospun fibrous mats are widely used in tissue engineering as scaffolds in biological substitutes.[29] PCL is one of the biodegradable poly ester approved by Food and Drug Administration (FDA), which has been used in biomedical applications.[30] However, PCL scaffolds have not formerly been used for cell culture applications involving tissue engineering due to its high hydrophobicity and poor cell adhesion. The individual problems associated with PCL can be limited by preparing graft polymers, blends and composites.[31] Blending PCL with other synthetic/natural polymers or other inorganic filler materials offers desired mechanical and biological properties.

Calcium sulfate (CaS*)*, as a commonly used implanting material, shows good biocompatibility, biodegradability, osteoconductivity and mechanical properties. Studies about using $CaSO_4$ as bone filler for the treatment of bone defects are reported now and then[17] but the fabrication of a synthetic polymer incorporated with calcium sulfate was hardly studied. Calcium sulfate in pure form is a biocompatible and osteo conductive material which could be an affordable material for bone regeneration applications. However, it has the disadvantages of low strength and fast resorption in vivo which limits its a pplication as bone grafts.[32] In this present study we used medical grade nano sized calcium sulfate along with PCL for fabricating electrospun fibrous mat.

Although numerous membrane materials have been investigated, few studies have focused on the technique of membrane fabrication. Hitherto most GTR/GBR membranes are made in the shape of porous foam, created by traditional methods such as particulate leaching, solvent casting or gas foaming. Recently, a new technique has been innovated, called electrospinning which allows to fabricate thin

nano-fibrous mats.[33] Porous electrospun mat was fabricated by using THF:DMSO as solvent for better spinnability.[34] The PCL and Calcium Sulfate - PCL solutions were electrospun in a horizontal spinning configuration, using a customized electrospinning unit (Holmarc, USA). Polymer solutions were supplied continuously to a syringe connected to a 21 gauge blunt end needle with a feeding rate of 1.5 ml/h for 4hr. A high voltage of 12 kv was applied between the needle kept at a distance of 16 cm from rotating mandrel set at 200 revolutions per minute. The feeding rate of 1.5ml/hr and voltage of 12kv is used in order to achieve uniform fiber distribution. Various parameters such as applied voltage, tip to collector distance, viscosity and concentration of solution can influence the morphology of fibers formed by electrospinning.[35] Since PCL is a unique polymer with good electrospinnability.

Mechanical and chemical properties of scaffolds were characterized using scanning electron microscopy(SEM), Fourier transform infra-red spectroscopy (FTIR), EDAX and Universal testing machine. The three dimensional(3D) morphological (fiber diameter and pore diameter, fiber distribution and pore distribution) and structural aspects of electrospun GTR mats was observed by scanning electron microscope. The PCL-CaS electrospun fibrous scaffold showed uniform fiber distribution. The elemental identification of calcium showed characteristic peak value ranging between 3.40 – 4.20$cm^{-1}$and sulfate showed characteristic peak value ranging between 1 – 2.60cm^{-1} which manifest the presence of calcium and sulfate groups [Graph 4]. The spectral analysis of CaS-PCL mat compared PCL mat and CaS powder showed bulk characteristic of PCL from the peaks at 1,720 cm^{-1} and 2,919 cm^{-1} for ester carbonyl group and C–H respectively[Graph 5 & 6].

The tensile strength of electrospun PCL scaffolds exhibited value is 5.564MPa and incorporation of 5%,10% calcium sulfate along with PCL exhibited as 3.122MPa (5%) and 4.236MPa (10%) respectively[Table 1]. Multiple comparison of tensile strength between the three groups have done. Statistically significant higher tensile strength was exhibited by the PCL membrane [5.56±0.70] when compared with other two groups [PCL+5%CaS and PCL+10%CaS]. The data exhibited by the other two membranes [PCL+5%CaS and PCL+10%CaS] was also statistically significant[Table 4,Graph1] . However the data of CaS-PCL is not compromised with PCL electrospun mat alone due to agglomeration of calcium sulfate in PCL scaffold, but it achieved a reasonable value with PCL. The suture retention strength of electrospun PCL scaffold is 0.19N ± 0.23 and where calcium sulfate incorporated PCL exhibited value is 0.41N ±0.26 (5%CaS) and 0.52N ±0.24 (10%CaS) respectively[Table 2].

Multiple comparison of suture pull out test between the three groups have done. Statistically significant higher suture pull out data was exhibited by PCL+10%CaS membrane [0.46±0.24*] when compared with other two groups [PCL and PCL+5%CaS][Table 5,Graph 2] . The physico-chemical properties of CaS-PCL blend scaffolds was further analyzed for its suitability for cell culture applications by analyzing the wettability of electrospun scaffolds. To the best of our knowledge no studies have evaluated the wettability of electrospun PCL –CaS until now. The PCL fibrous mat showed water contact angle of 130^0 ± 4.72, whereas 5% CaS-PCL blend fibrous mat expressed lower values of 128^0± 2 and 10% CaS-PCL blend expressed 118^0 ±1.5[Table 3]. Multiple comparison of water contact angle values between the three groups have done. Statistically significant higher water contact angle data was

exhibited by PCL+10%CaS membrane [119.67±1.52*,#] when compared with other two groups [PCL and PCL+5%CaS] [Table 6,Graph 3].This confirms that PCL-CaS mats are relatively more hydrophilic compared to that of PCL.

The electrospun scaffolds for tissue engineering should be porous with adequate surface chemistry and biocompatibility.[36] The biocompatibility of electrospun PCL-CaS fibrous mats was evaluated in vitro by direct contact assay and cell adhesion. The test material PCL, PCL CaS achieved a numerical grade not greater than 2 (Mild Zone limited to area under specimen), the material is considered as not cytotoxic. Negative control gave none cytotoxic reactivity and positive control gave severe cytotoxic reactivity as expected. The cell adhesion result showed L-929 cells were adhered and well spread on test samples of PCL and PCL-CaS as well. Hence the morphological evaluation of PCL-CaS electrospun scaffold showed no cytotoxic effect and well adhering property towards L929cells. These results indicates that PCL-CaS electrospun scaffolds were superior compared to PCL and suitable for periodontal tissue engineering.

Even though the results of present study showed that the elecrospun GTR membranes possessed appropriate invitro properties and excellent cytoskeletal architechture, there are certain limitations of the study. The cell culture analysis of the study here was of limited and in the future the membranes should be completely analysed using different cell types such as gingival fibroblast , periodontal ligament fibroblasts and osteoblast like cells in terms of cell attachment , proliferation, migration and differentiation. Another limitation is that the present study have not done any degradation aspect which is a remarkable limitation of PCL.

SUMMARY

In the present study PCL incorporated with bioactive calcium sulfate composite membranes were fabricated by electrospinning method and their in-vitro properties were characterized. All the electrospun fibrous membranes exhibited appropriate mechanical and good chemical properties. Moreover none of the membranes found to be cytotoxic but it possess excellent cytoskeletal architecture and cell spreading.

On comparing the overall properties PCL +10% CaS found to be superior compared to other membranes. In case of tensile strength and suture pull out test, PCL-CaS electrospun fibrous mat exhibited appropriate value with PCL and wettability of PCL-CaS electrospun mat showed relatively more hydrophilicity compared to that of PCL. The cell adhesion result showed L-929 cells were adhered and well spread on test samples of PCL and PCL-CaS and exhibited a required cytocompatibility.

CONCLUSION

PCL- CaS blend was prepared in a solvent of THF and DMSO and fabricated by employing electrospinning technique. In the present study the results showed that composite electrospun scaffolds (PCL-CaS) were superior to PCL scaffolds. Based on the above results, we conclude that the electrospun mat fabricated with PCL incorporated with medical grade Calcium sulfate employed by electrospinning technique is suitable for periodontal tissue engineering. Further animal experiments followed by human clinical trials need to be evaluated for future applications

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ANNEXURES

Annexure 1

Tele : 91 471 2340801 Fax : 91 471 2341814

श्री चित्रा तिरुनाल आयुर्विज्ञान तथा प्रौद्योगिकी संस्थान
बायो मेडिकल टैक्नोलॉजी विंग, पूजापुरा, तिरुवनन्तपुरम-695 012, इन्डिया

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
BIO MEDICAL TECHNOLOGY WING POOJAPPURA, THIRUVANANTHAPURAM-695 012, INDIA
(An Institute of National Importance under Govt. of India)

Date : 18 July 2016

Dr.Manoj Komath, Ph D
Scientist F, Bioceramics Division

To

Dr.Elizabeth Koshi
Principal
Sree Mookambika Institute of Dental Sciences
Kulashekharam

Sub : Permission for PG students to do research studies.

Sir/Madam,

As per your request dated 12.07.2016, I hereby promise to allow your PG student Dr. Anju Unnikrishnan (Dept of Periodontics and Implantology) to conduct experiments in our lab, which are essential for her research work entitled "Fabrication and in vitro Characterization of Electrospun PCL Mats Containing Bioactive Calcium Sulfate for Periodontal Tissue Regeneration".

This will be done as an academic interaction, on collaborative terms and conditions between the Institutions.

Thanking you,

Sincerely,

(Manoj Komath)

Annexure 3

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

KULASEKHARAM, KANYAKUMARI DIST., TAMIL NADU, INDIA.

INSTITUTIONAL RESEARCH COMMITTEE

Certificate

This is to certify that the research project protocol, ***Ref no. 09/07/2016*** titled, ***"Fabrication and in-vitro characterization of electrospun polycaprolactone mats containing bioactive calcium sulfate for periodontal tissue regeneration"*** submitted by ***Dr. Anju Unnikrishnan, II Year MDS, Department of Periodontics*** has been approved by the Institutional Research Committee at its meeting held on ***26th July 2016.***

Convener
Dr. T. Sreelal

Secretary
Dr. Pradeesh Sathyan

Annexure 3

INSTITUTIONAL HUMAN ETHICS COMMITTEE

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES,
KULASEKHARAM, TAMILNADU

Communication of Decision of the Institutional Human Ethics Committee(IHEC)

SMIMS/IHEC No:1 /Protocol no: 12 / 2016

Protocol title: Fabrication and in- vitro characterization of electrospun polycaprolactone mats containing Bioactive calcium sulfate for periodontal tissue regeneration
Principal Investigator: Dr. Anju Unnikrishanan
Name& Address of Institution: Department of Periodontics, Sree Mookambika Institute of Medical Sciences, Kulasekharam ☑ New review ☐ Revised review ☐ Expedited review
Date of review (D/M/Y): Date of previous review , if revised application:
Decision of the IHEC: ☑ Recommended ☐ Recommended with suggestions ☐ Revision ☐ Rejected
Suggestions/ Reasons/ Remarks:
Recommended for a period of :six months

Please note*

- Inform IHEC immediately in case of any Adverse events and Serious adverse events.
- Inform IHEC in case of any change of study procedure, site and investigator
- This permission is only for period mentioned above. Annual report to be submitted to IHEC.
- Members of IHEC have right to monitor the trial with prior intimation.

Reneegalgangadhar

Signature of Member Secretary IHEC

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES ★ KULASEKHARAM ★
IHEC
Date 21/12/16